Bile salt and cholesterol metabolism in diabetes mellitus type 1

Bakker-van Waarde, Willemijntje Maria

Bile salt and cholesterol metabolism in diabetes mellitus type 1

Willie M. Bakker-van Waarde

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Bile salt and cholesterol metabolism in diabetes mellitus type 1

Willemijntje Maria Bakker-van Waarde

Groningen, 08-02-2006

1. Een vertraagde chylomicronenklaring als risicofactor voor atherosclerose is bij kinderen met diabetes mellitus type 1 op late tienerleeftijd niet aantoonbaar. Dit proefschrift
2. Diabetes mellitus type 1 leidt bij kinderen en volwassenen tot een verlaging van de cholesterol synthese, waarschijnlijk als gevolg van een toegenomen cholesterolabsorptie. Gylling H et al., Diabetes 2004;53:2217-22 en dit proefschrift
3. Bij door streptozotocine-geïnduceerde diabetes in de rat is de relatief verminderde uitscheiding van cholesterol in de gal en de verhoogde cholesterolabsorptie, ten minste gedeeltelijk, toe te schrijven aan een verlaagde expressie van Abcg5 en Abcg8 in lever en darm. Dit proefschrift
4. De sterk verhoogde maximale secretiesnelheid van galzouten bij experimentele diabetes wordt niet verklaard door inductie van Abcb11 (Bsep) expressie, maar waarschijnlijk door de sterk verhoogde Abcb4 (Mdr2) expressie. Dit proefschrift
5. De verlaging van de galzout-onafhankelijke galflow bij experimentele diabetes wordt veroorzaakt door een verlaagde Abcc2 (Mrp2) expressie en een daardoor afgenomen glutathione uitscheiding. Dit proefschrift
6. Bij ratten met door streptozotocine-geïnduceerde diabetes is de toegenomen cholaat poolgrootte het gevolg van zowel een verhoogde cholaatsynthese als een verhoogde cholaatabsorptie. Dit proefschrift
7. De effectiviteit en veiligheid van lipiden-verlagende medicatie voor de preventie van hartvaatziekten bij kinderen met type 1 diabetes en de leeftijd waarop deze behandeling zou moeten worden gestart dient wetenschappelijk te worden onderzocht.
8. Insulinepompbehandeling bij jonge kinderen met diabetes mellitus type 1 is een effectieve methode voor het verbeteren van de glycemische controle en kan het risico van ernstige hypoglycaemieën in deze leeftijdsgroep verlagen. Weinzimmer SA et al., Pediatrics 2004;114:1601-5
9. Bij kinderen met diabetes mellitus type 1 en insulinepomp behandeling is een beter HbA1c geassocieerd met een pompinstelling, waarbij minder dan 50% van de dagelijkse insuline hoeveelheid via de basale snelheid wordt toegediend en frequent een insulinebolus wordt gegeven. Danne T et al., poster ADA 2005
10. Een kinderarts wordt opgeleid in het geven van behandeladviezen aan kinderen met een chronische ziekte, echter, om een gedragsverandering te bewerkstelligen die leidt tot het opvolgen van deze behandeladviezen, schiet de opleiding regelmatig tekort.
11. De beste manier om iets af te krijgen is te beginnen. E van Waarde-Broere
12. Statistics are no substitute for judgement. H Clay
13. Een "brug" is zowel mogelijk tussen ziekenhuis en faculteit als tussen klinisch en basaal wetenschappelijk onderzoek.
14. Fat is the largest endocrine gland and it keeps getting larger. JJ Van Wijk
15. When a problem is too large and seems unsolvable, don't forget that you can eat an elephant assuming it is cut into small enough pieces. R Hanas, Insulin-dependent diabetes in children, adolescents and adults 1998.

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Voor mijn vader en moeder
Voor Martin en Lotte
en speciaal voor Elianne

Paranimfen: Klasien A. Bergman
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Contents

1 General Introduction and Outline of the Thesis

INTRODUCTION

Type 1 diabetes mellitus is an autoimmune disease resulting from T-cell-mediated destruction of the insulin-producing beta cells in the pancreas[1]. Its etiology is highly complex, involving both environmental and genetic factors. The disorder is characterized by progressive loss of beta cell function and the development of hyperglycemia and ketoacidosis in the absence of adequate insulin treatment. Type 1 diabetes is associated with microvascular complications, such as retinopathy, nephropathy and neuropathy. Chronic hyperglycemia seems to be the major initiating factor of microvascular disease. Type 1 diabetes is also associated with macrovascular disease, which is the main cause of death in this patient group. In the Framingham study, the cumulative coronary artery disease mortality in patients with type 1 diabetes at the age of 55 years was four times that of the same age group without diabetes[2]. Roper et al.[3] showed even higher mortality rates from cardiovascular disease in patients with type 1 diabetes, especially in women. In children with type 1 diabetes, atherosclerotic processes probably commence in association with the onset of diabetes. Several studies have demonstrated early

Table 1. Possible mechanisms by which hyperglycemia may affect macrovascular complications in type 1 diabetes.

Mechanisms	Pathways and results	Results
↑ oxidative stress	↑ cytokines (IL-1, TNFalpha) ↑ cytoadhesive proteins (ICAM-1, E-sel) procoagulate state (PAI-1, VWF) aldose reductase-polyol pathway	↑ oxidative stress
	AGE formation	↓ elasticity blood vessels ↑ cytokines procoagulate state (TF, VCAM-1)
	hexosamine pathway protein kinase C activation	↑ PAI-1 ↓ NO ↑ endothelin-1 ↑ PAI-1
↓ NO production and NO responsiveness	platelet aggregation ↑ MCP-1 ↑ P-selectin and VCAM-1	
Glycosylation of lipoproteins	↓ clearance ↑ uptake by macrophages (foam cells)	

AGE: advanced glycation end products, E-sel: E-selectin, ICAM-1: intercellular adhesion molecule-1, IL-1: interleukin-1, MCP-1: monocyte chemoattractant protein-1, NO: nitric oxide, TNFα: tumor necrosis factorα, PAI-1: plasminogen activator-inhibitor-1, TF: tissue factor, VCAM-1: vascular cell adhesion molecule-1, VWF: von Willebrand factor

signs of atherosclerosis in children and adolescents with type 1 diabetes[4-7]. Insight in the factors contributing to the development of diabetes-associated atherosclerosis is important to facilitate development of novel strategies to prevent progression of this important complication of diabetes.

Pathophysiologic features of macrovascular complications

Chronic hyperglycemia may lead to endothelial dysfunction in type 1 diabetes (Table 1). Hyperglycemia induces formation of reactive oxygen species[8,9]: increased oxidative stress will enhance the production of cytokines and cytoadhesive proteins and may induce a procoagulate state by stimulation of plasminogen activator-inhibitor 1 (PAI-1) and von Willebrand factor production. Overproduction of superoxide, in turn, may induce several pathophysiologic pathways responsible for diabetic complications, such as AGE formation and protein kinase C activation (Table1)[10,11]. In addition, hyperglycemia may inhibit vascular nitric oxide (NO) production[12] and the responsiveness to NO[13]. Decreased NO activity may lead to platelet aggregation, to increased expression of monocyte chemoattractant protein-1 (MCP-1) and to the expression of adhesion molecules (Table1). Surprisingly, improvement in glycemic control is not always associated with a decrease in macrovascular complications[14-18]. Other risk factors for CVD in type 1 diabetes are age, waist-to-hip ratio, blood pressure, albumin excretion rate, smoking and plasma lipid levels[16,17]. Research described in this thesis is mainly focused on factors contributing to altered lipid metabolism in type 1 diabetes.

Lipoprotein metabolism and the effect of type 1 diabetes

Elevated plasma lipids constitute an established risk factor for cardiovascular disease. Triglycerides (TG), cholesterol (esters) and phospholipids (PL) are transported in lipoproteins. The hydrophobic triglycerides and cholesteryl esters comprise the core of the lipoproteins and are enveloped by the amphipathic phospholipids, free cholesterol and several proteins. The proteins, called apoproteins or apolipoproteins, are critical regulators of lipid transport. Lipoproteins have traditionally been subdivided on the basis of their density, which is inversely related to their size and lipid content. There are four major classes of lipoproteins, i.e., chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). LDL and HDL are the major carriers of cholesterol (esters), while chylomicrons and VLDL are the major carriers of triglycerides.

Transport of exogenous (dietary) lipids

TG and PL are very efficiently absorbed from the intestine (>90%), whereas the absorption of cholesterol averages ~ 50% in humans. TG are hydrolyzed by lipases into free fatty acids and small amounts of monoglycerides and diglycerides

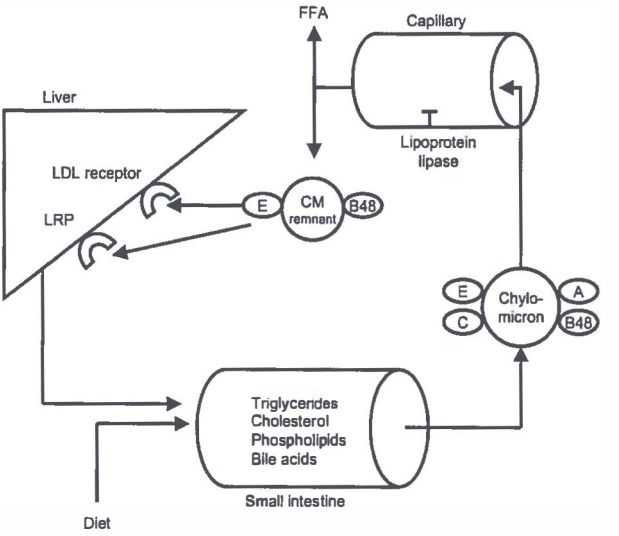

Figure 1: Schematic overview of the transport of exogenous (dietary) lipids.
A: apolipoprotein-A, B48: apolipoprotein-B48, C: apolipoprotein-C, CM: chylomicron, E: apolipoprotein-E, FFA: free fatty acid, LDL: low density lipoprotein, LRP: LDL receptor-related protein.

in the gastrointestinal lumen. After uptake by the enterocytes, reesterification to TG occurs. Triglycerides, cholesterol, phosholipids, apo-B48, apo-AI and apo-AIV are assembled into chylomicrons. Microsomal triglyceride transfer protein (MTP) is necessary for transferring triglycerides, cholesterol and phospholipids to apo-B-containing primordial particles (i.e., VLDL and chylomicrons)[19]. Following the secretion of chylomicrons into lymph and their subsequent entry into the bloodstream via the thoracic duct, they acquire apo-E and apo-C, both originating from HDL. Chylomicron triglycerides are hydrolyzed by lipoprotein lipase (LPL), which resides on the surface of capillary endothelial cells. The remaining chylomicron remnant will transfer excess surface phospholipids, cholesterol, apo-A and apo-C to HDL. The chylomicron remnant, which is cholesteryl ester-enriched, will be removed rapidly from the circulation by the liver. At the hepatic surface sequestration occurs within the space of Disse, which involves binding to heparan sulfate proteoglycan (HSPG), possibly mediated by apoE. LPL and hepatic lipase may continue processing the remnants to form smaller particles. Uptake of the chylomicron remnants by hepatocytes involves both low-density lipoprotein receptor, in a process mediated by apo-E, and HSPG/LDL receptor-related protein (LRP)(Figure 1)[20].

Transport of exogenous (dietary) lipids in type 1 diabetes mellitus

Insulin deficiency is associated with decreased LPL activity[21] and increased expression of apo-CIII[22]. Apo-CIII may displace apo-E from lipoproteins, leading to

impaired hepatic uptake of chylomicrons[23]. In adult patients with type 1 diabetes, a decreased clearance of chylomicrons has been described which appeared to result from decreased hepatic uptake rather than from impaired lipolysis[24]. A recent study by Ebara et al. in mice with streptozotocin-induced diabetes showed a delayed chylomicron clearance rate in the diabetic state associated with a decrease in HSPG production[25]. Delayed clearance of postprandial lipoproteins and the accumulation of cholesterol-enriched remnant particles have been associated with the development of cardiovascular disease[26,27,28]. It has been shown that chylomicron remnants are more cholesterol-enriched in type 1 diabetes than in controls[24].

Transport of endogenous lipids / the apo-B100 lipoprotein system

VLDL particles are formed and secreted by the liver. VLDL-TG derive from remodeled chylomicron remnant TG, free fatty acids flux from adipose tissue to the liver and, to a relatively small extent, from de novo lipogenesis. VLDL contain apo-B100, apo-C and apo-E. Like chylomicrons, VLDL-TG are hydrolyzed by endothelial LPL and hepatic lipase. As lipolysis proceeds, VLDL become smaller and denser to become VLDL remnants and, subsequently, IDL. VLDL remnants and IDL can be taken up by the liver via the LDL receptor or LRP mediated by apo-E. Furthermore, direct uptake of VLDL remnants / IDL in various tissues via the LDL receptor (apo-

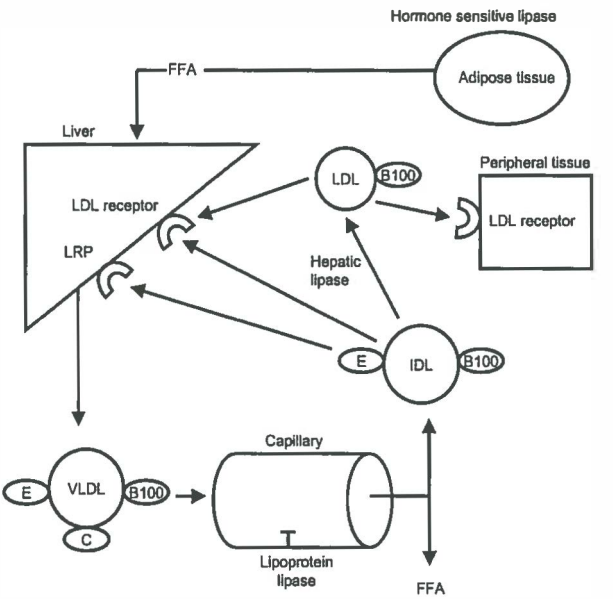

Figure 2: Schematic overview of the transport of endogenous lipids / the apoB-100 lipoprotein system. B100: apolipoprotein-B100, C: apolipoprotein-C, E: apolipoprotein-E, FFA: free fatty acid, IDL: intermediate density lipoprotein, LDL: low density lipoprotein, LRP: LDL receptor-related protein, VLDL: very low density lipoprotein.

B/E) can take place. Alternatively, IDL can be converted to form LDL. LDL contains cholesterol esters as the major lipid and apo-B100 as its sole apolipoprotein. LDL particles can therefore only be cleared via the LDL receptor, with apo-B100 acting as ligand. Apo-E containing lipoproteins have 20-fold greater affinity for the LDL receptor than apo-B100-containing LDL[29,30]. Clearance of LDL is much slower (half life 2-3 days) compared to VLDL remnants (minutes-hours). Long term residence of LDL in blood increases its chance of modification through oxidation and/or glycation. Modification of LDL prevents its recognition by the LDL receptor and causes uptake of the modified LDL in macrophages via scavenger receptors and, hence, in foam cell formation.

Transport of endogenous lipids / the apoB-100 lipoprotein system in type 1 diabetes mellitus

In case of insulin deficiency, impaired action of LPL will lead to elevated VLDL triglyceride levels. Insulin deficiency will lead to increased FFA release from adipose tissue to the liver by the derepressed action of hormone sensitive lipase. The increased FFA flux towards the liver is thought to stimulate VLDL production. Plasma LDL cholesterol levels may also be elevated, via increased LDL production from VLDL and diminished insulin-stimulated, LDL receptor-mediated uptake[31]. Clearance of VLDL remnants may also be impaired[32]. In patients with adequate glycemic control, plasma lipid levels are generally normal or even favorable, although lipoprotein composition abnormalities can persist[33]. VLDL and LDL particles are enriched with free and esterified cholesterol in diabetes[34,35,36] and a preponderance of small dense LDL has been found[37]. In addition to glycosylation, oxidation of lipoproteins is enhanced in diabetes and may accelerate the development of atherosclerosis[8].

The reverse cholesterol (HDL) pathway

HDLs originate from liver, intestine and from shedded surface material of chylomicrons and VLDL during lipolysis (primarily apo-AI and phospholipid). The last process is facilitated by the phospholipid transfer protein (PLTP)[38]. The ATP binding cassette transporter A1 (ABCA1) facilitates the transfer of cholesterol and phospholipids from cells to pre-β-HDL[39,40,41] and is essential for HDL formation. Free cholesterol is converted to cholesteryl ester by lecithin:cholesterol acyltransferase (LCAT), resulting in small spherical mature HDL particles (HDL_3). HDL_3 also serves as acceptor for free cholesterol. The enlargement of HDL_3 upon acquiring cholesteryl-esters leads to the formation of HDL_2. Some HDL_2 particles can be further enriched with cholesterol and also receive apo-E, leading to the formation of HDL_1. The catabolism of HDL is not entirely understood. HDL_1 can bind to the LDL receptor or to LRP via apo-E and be metabolized. The cholesterol ester transfer protein (CETP) transfers cholesteryl esters from HDL_2 to VLDL, LDL, IDL

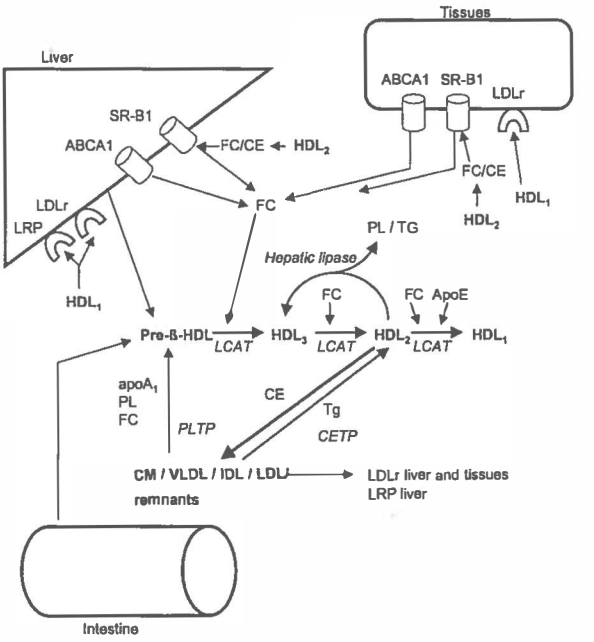

Figure 3: Schematic overview of the reversed cholesterol (HDL) pathway. ABCA1: ATP binding cassette transporter A1, apoA1: apolipoprotein-A1, apoE: apolipoprotein-E, CE: cholesteryl ester, CETP: cholesterol ester transfer protein, CM: chylomicron, FC: free cholesterol, HDL: high density lipoprotein, IDL: intermediate density lipoprotein, LCAT: lecithin:cholesterol acyltransferase, LDL: low density lipoprotein, LDLr: LDL receptor, LRP: LDL receptor-related protein, PL: phospholipids, PLTP: phospholipid transfer protein, SR-B1: scavenger receptor class B type 1, TG: triglycerides, VLDL: very low density lipoprotein.

and remnants and in exchange transfers triglycerides from these lipoproteins to HDL. In humans, the CETP pathway is the main route for delivering cholesterol to the liver. Rats and mice do not have CETP. Hydrolysis of triglycerides in HDL_2 by hepatic lipase will lead to the re-formation of HDL_3[42]. The scavenger receptor class B type 1 (SR-B1) mediates bi-directional flux of cholesterol from cells to HDL[43,44]. Binding of HDL_2 to SR-B1 at the cellular membrane enables selective uptake of cholesterol and cholesteryl ester by cells.

The reversed cholesterol (HDL) pathway in type 1 diabetes mellitus

In patients with type 1 diabetes in poor control, plasma HDL concentrations are low compared to non-diabetic controls but rise with improvement of glycemic control[45]. Glycosylation of lipoproteins may lead to an increased transfer of esterified cholesterol from HDL to triglyceride-rich lipoproteins[46]. In type 1 diabetes a decrease in cholesteryl ester / triglyceride ratio in HDL has been found[36]. Furthermore, phospholipid composition of HDL may be altered in type 1 diabetes, which may affect reverse cholesterol transport[47]. In vitro studies showed

that AGE-modified proteins affect SR-B1 and thereby inhibit the selective uptake of HDL-cholesteryl esters as well as cholesterol efflux from peripheral cells to HDL[48]. In the liver and in peritoneal macrophages of streptozotocin-diabetic mice, ABCA1 gene expression was found to be decreased and to be restored upon insulin treatment[49].

Studies on atherosclerosis and lipid levels in children with type 1 diabetes

Several studies have shown early signs of atherosclerosis in children and adolescents with type 1 diabetes, such as impaired endothelium-dependent vasodilatation and increased carotid artery intima-media thickness[4,5,6]. Signs of oxidative stress and increased levels of AGEs were found in children with type 1 diabetes[50,51,52]. Most studies revealed the presence of increased plasma levels of total cholesterol, LDL cholesterol and triglycerides in children with type 1 diabetes[53-56]. Plasma lipid levels were associated with glycemic control (HbA_{1c}) and with lipid concentrations in the parents[54].

Cholesterol and bile salt synthesis and transport

Hepatic conversion of cholesterol into bile salts and biliary excretion of cholesterol for eventual disposal in stool are quantitatively the most important pathways for removal of excess cholesterol from the body[57]. Bile salts facilitate the intestinal absorption of dietary fat, fat-soluble vitamins and cholesterol, and, thereby, modulate the level of expression of LDL receptors in the liver[58]. Bile salts repress their own biosynthesis, thereby decreasing cholesterol turnover. Recently, several transcription factors and transport proteins involved in bile salt and cholesterol turnover have been identified. Data on hepatic cholesterol and bile salt excretion and intestinal uptake in type 1 diabetes are limited and were the focus of most studies described in the thesis.

Cholesterol synthesis and enterohepatic circulation

Cholesterol is synthesized from acetate moieties. Three molecules of acetate form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). HMG-CoA is converted to mevalonic acid by HMG-CoA reductase. Through a series of steps, mevalonic acid is converted to cholesterol. HMG-CoA reductase catalyzes an important rate-controlling step in cholesterol biosynthesis. Cholesterol can not be eliminated by catabolism; it must be excreted into bile as cholesterol or as bile salts. About 50% of biliary and dietary cholesterol is reabsorbed from the intestine and recirculates back to the liver[59].

Bile salt synthesis and enterohepatic circulation

Approximately 500 mg of cholesterol is converted into bile salts each day in the

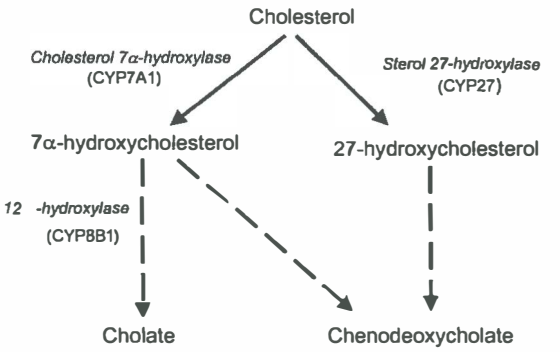

Figure 4: Simplified scheme of bile salt synthesis. The conversion of cholesterol into 7α-hydroxycholesterol by cholesterol 7α-hydroxylase (Cyp7A1) is the first step of the neutral or classical pathway of bile salt synthesis leading ultimately to the formation of cholate and chenodeoxycholate. Sterol 27-hydroxylase (Cyp27) catalyses the conversion of cholesterol to 27-hydroxycholesterol, initiating the acidic or alternative pathway and ultimately leading to the formation of chenodeoxycholate. Sterol 12α-hydroxylase (Cyp8B1) controls the ratio cholate chenodeoxycholate.

adult human liver. Formation of bile salts from cholesterol involves 17 different enzymes, many of which are exclusively expressed in the liver. The immediate products of these pathways are referred to as primary bile salts[60]. Cholesterol 7α-hydroxylase (Cyp7A1), sterol 27 hydroxylase (Cyp27) and sterol 12α-hydroxylase (Cyp8B1) are key enzymes in bile salt synthesis (Figure 4). Cyp7A1 catalyses the conversion of cholesterol into 7α-hydroxycholesterol, the first step of the so-called neutral pathway of bile salt synthesis that yields both primary bile salts, i.e., cholate and chenodeoxycholate. Cyp27 catalyses the conversion of cholesterol to 27-hydroxycholesterol, initiating the acidic pathway of bile salt synthesis that will ultimately lead to the formation of chenodeoxycholate in humans and hydroxylated forms hereof, muricholates, in rodents[60]. Cyp8B1 is essential for the formation of cholate from 7α-hydroxycholesterol and controls the ratio in which the primary bile salts cholate and chenodeoxycholate are being formed[61]. Anaerobic bacteria present in the gut modify bile salt structures into secondary bile salts, *e.g.*, lithocholate and deoxycholate. In the enterohepatic circulation about 95% of the bile salts are reabsorbed from the intestine and return to the liver for resecretion into the bile. A relatively small fraction of bile salts is lost in the faeces and this loss equals *de novo* synthesis in the liver[62].

Hepatic and intestinal transport of cholesterol (Figure 5 and 6)
Hepatic uptake of cholesterol from chylomicrons, VLDL, IDL, LDL and HDL is performed by the receptors earlier described (i.e., LDL receptor, LRP, SR-B1). Cholesterol can be secreted from the liver into plasma as VLDL or, via ABCA1 and

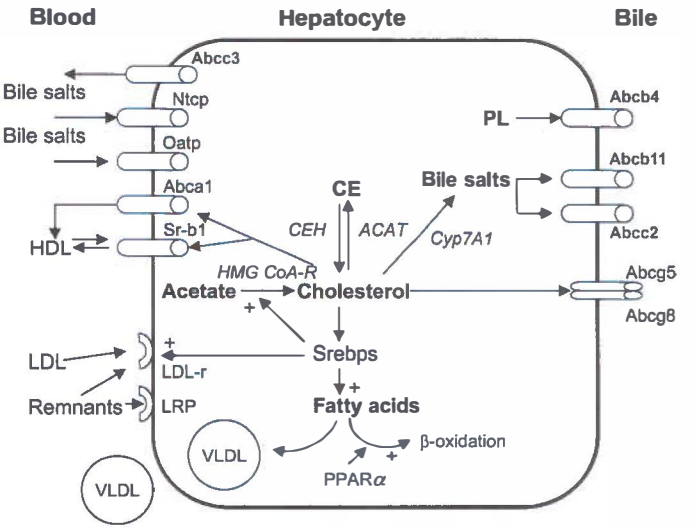

Figure 5: Schematic overview of bile salt and cholesterol transport in hepatocytes. Uptake of bile salts into hepatocytes at the basolateral surface (from the sinusoids) can take place via Na^+-taurocholate cotransporting polypeptide (Ntcp, Slc10a1) and via several members of the organic anion-transporting polypeptide (Oatp) family of transporters. Bile salts may be resecreted from the hepatocyte into the blood via Abcc3 (multidrug-resistance protein 3 or Mrp3). In the hepatocyte bile salts can be synthesized from cholesterol, with cholesterol 7α-hydroxylase (Cyp7A1) as first enzyme in the neutral pathway. Bile salts will be secreted from the hepatocyte into bile predominantly via Abcb11 (bile salt export pump or Bsep) and the glucuronidated and sulfated bile salts via Abcc2 (multidrug resistance-associated protein 2 or Mrp2).
Cholesterol uptake from chylomicrons, VLDL, IDL, LDL and HDL can take place via the low density lipoprotein receptor (LDL-r), LDL receptor-related protein (LRP) and scavenger receptor class B type 1 (Sr-b1). In the hepatocyte, cholesterol may be synthesized from acetate with hydroxy-methylglutaryl coenzyme A reductase (HMG CoA-R) as rate-limiting step. Storage of cholesterol in the hepatocyte is mediated by Acyl-Coenzyme A:Cholesterol Acyltransferase (ACAT) leading to the formation of cholesteryl esters. Cholesteryl esters can be hydrolyzed to generate free cholesterol by cholesterol ester hydrolase (CEH). Secretion of free cholesterol from the hepatocyte into the blood may involve the ATP binding cassette transporter A1 (Abca1) and Sr-b1 towards HDL and possibly under influence of microsomal triglyceride transfer protein towards VLDL. Biliary cholesterol secretion is mediated by the ATP binding cassette transporters G5 and G8 (Abcg5, Abcg8). Cholesterol secretion into bile has been shown to be coupled to phospholipid secretion in part controlled by bile salt secretion. Abcb4 (multidrug resistance P-glycoprotein type 2 or Mdr2) controls biliary phospholipid secretion. Sterol regulatory element-binding proteins (Srebps) are transcription factors that are activated by low intracellular cholesterol concentrations. Srebp-1c enhances transcription of genes encoding enzymes involved in free fatty acid synthesis. The formed fatty acids may be secreted as triglycerides in very low density lipoproteins (VLDL), or metabolized in the β-oxidation under influence of the transcription factor peroxisome proliferator-activated receptor α (PPARα). Srebp-2 may activate HMG CoA-reductase and stimulate LDL receptor expression.

possibly SR-B1, as HDL. Cholesterol secretion into bile is coupled to phospholipid secretion in a process that is, in part, controlled by bile salt secretion[63]. Biliary phospholipid secretion is controlled by Abcb4 (multidrug resistance P-glycoprotein type 2 or Mdr2-Pgp) in rodents, and by its homologue (ABCB4) MDR3 in humans[64,65]. The ATP binding cassette halftransporters Abcg5 and Abcg8 are involved in transport of cholesterol from the hepatocyte into bile and from the enterocyte into the intestinal lumen[66,67,68]. Niemann-Pick C1 Like 1 (NPC1L1) protein is critical for the uptake of cholesterol from the intestine into the enterocyte[69,70]. SR-B1 may also play a role in intestinal cholesterol absorption[43] but its exact role has remained unclear sofar.

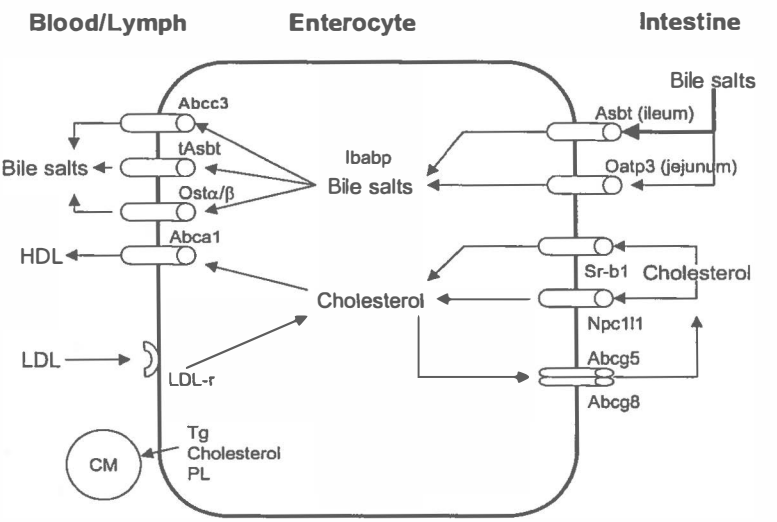

Figure 6: Schematic overview of bile salt and cholesterol transport in enterocytes.
Intestinal reabsorption of bile salts occurs primarily in the ileum via the apical sodium-dependent bile salt transporter (Asbt). In the jejunum Na^+-independent bile salt uptake is mediated by the organic anion transporting polypeptide 3 (Oatp3). Intracellular transport of bile salts in the enterocyte is mediated by the ileal bile acid-binding protein (Ibabp). Basolateral extrusion of bile salts into the blood can be mediated by Abcc3 (multidrug-resistance protein 3 or Mrp3), the truncated isoform of Asbt (t-Asbt) and the organic solute transporter α and β (Ost-α/β). Intestinal cholesterol absorption involves the action of Niemann-Pick C1 Like 1 (Npc1l1) protein and, possibly, of scavenger receptor class B type 1 (Sr-b1). The ATP binding cassette transporters G5 and G8 (Abcg5/Abcg8) mediate resecretion of cholesterol from the enterocyte into the intestine. At the basolateral membrane, cholesterol may enter the enterocyte via the LDL receptor (LDL-r). Cholesterol may be secreted from the enterocyte towards HDL via ATP binding cassette transporter A1 (Abca1). Cholesterol together with triglycerides, phospholipids, apo-B48, apo-A1 and Apo-AIV are assembled into chylomicrons and secreted into lymph.

Hepatic-, cholangiocyte- and intestinal transport of bile salts (Figure 5 and 6)

The entry of bile salts into hepatocytes at their basolateral surface (in contact with the blood sinusoids) occurs by at least two processes. The major uptake system is represented by Na^+-taurocholate-cotransporting polypeptide (NTCP, *SLC10A1*) that mediates a sodium-dependent transport process driven by the transmembrane Na^+ gradient maintained by Na^+/K^+-ATPase activity. A second Na^+-independent uptake system is mediated by several members of the organic anion-transporting polypeptide (OATP) family of transporters, which function as anion exchangers[71,72,73]. Canalicular transporters mediate the transport of bile salts from the hepatocyte into bile. Conjugated bile salts are excreted into bile via Abcb11 (bile salt export pump or Bsep). Abcc2 (multidrug resistance-associated protein 2 or Mrp2) is involved in biliary secretion of glucuronidated and sulfated bile salts. Abcc2 excretes several components including conjugated bilirubin, glutathione and many drugs[71,72,73].

Cholangiocytes are capable of Na^+-dependent uptake of bile salts from the bile via the apical sodium-dependent bile salt transporter Asbt (Slc10a2), allowing intrahepatic cycling of bile salts. After uptake in the cholangiocyte, bile salts are effluxed across the basolateral cholangiocyte membrane into the periductular capillary plexus. The organic anion transporting polypeptide A (OATP-A, SLC21A3), Abcc3 (multidrug-resistance protein 3 or Mrp3), and/or the truncated isoform of Asbt (t-Asbt) may be involved in the efflux of bile salts across the basolateral cholangiocyte membrane[71,73]. Cholangiocytic uptake may be relevant for the process termed cholehepatic shunting of bile salts, which, in theory, enhances the choleretic activity of these molecules.

Intestinal reabsorption of bile salts occurs primarily in the ileum via Asbt, also known as ileal bile salt transporter or ileal sodium-dependent bile salt transporter. In addition, Na^+-independent uptake is mediated by Oatp3 (*Slc21a7*), shown to be expressed at the apical surface of jejunal enterocytes. Basolateral extrusion of bile salts into the capillaries of the venous portal system can be mediated by Abcc3 (Mrp3) , t-Asbt or the organic solute transporters alpha and beta (Ost-α/β)[71-74].

Transcription factors involved in cholesterol and bile salt metabolism

Cholesterol and bile salt metabolism are influenced through the actions of several transcription factors. A detailed description of their modes of action and characteristics is beyond the scope of this introduction, only general aspects related to the work described in this thesis will be provided. For further details, the reader is referred to the specific references. A first important group are membrane-bound transcription factors designated sterol regulatory element-binding proteins (SREBPs), that activate the expression of more than 30 genes involved in the production of lipids for export into the plasma as lipoproteins and into the bile

as micelles. In addition, several nuclear receptors have been found to impact cholesterol and bile salt metabolism.

Sterol regulatory element-binding proteins (SREBPs)

SREBPs, in general, control genes encoding enzymes involved in fatty acid and cholesterol synthesis. SREBPs are activated by low intracellular cholesterol levels. When cells become depleted in cholesterol, SREBP cleavage-activating protein (SCAP) (a sensor for intracellular sterol levels) escorts SREBP from the endoplasmic reticulum to the Golgi apparatus. In the Golgi apparatus further processing by proteases will lead to the formation of nuclear SREBP (nSREBP). After translocation to the nucleus, nSREBP activates transcription of multiple target genes by binding to sterol response elements (SREs)[75]. There are three SREBP isoforms, designated SREBP-1a, SREBP-1c and SREBP2. SREBP-1a and SREBP-2 are the predominant isoforms of SREBP in most cultured cell lines, whereas SREBP-1c and SREBP2 predominate in the liver and most other tissues in the in vivo situation[76]. SREBP-1c preferentially enhances transcription of genes encoding enzymes involved in fatty acid synthesis and in triglyceride and phospholipid synthesis. SREBP2-preferentially activates genes encoding enzymes involved in cholesterol synthesis, e.g., HMG-CoA reductase. SREBP-1a activates all SREBP responsive genes (see for review[77,78]). SREBP-1a and SREBP2 also induce LDL receptor expression[79]. The promoter of SREBP-1c contains response elements for insulin and glucagon. Insulin stimulates SREBP-1c expression, leading to enhanced fatty acid synthesis[80]. Glucagon decreases the mRNA for SREBP-1c[81]. SREBP-1c mRNA levels falls in streptozotocin-induced diabetic rats and rises after insulin injection[82]. SREBP-1c may also contribute to the regulation of glucose metabolism by inducing the expression of glucokinase, a key enzyme in glucose utilisation, and by suppressing phosphoenolpyruvate carboxykinase, a key gluconeogenic enzyme[83,84].

Nuclear receptors

The activity of nuclear receptors is controlled by the binding of endogenous ligands like hormones, fatty acids, bile acids and oxysterols, or exogenous ligands, such as drugs and xenobiotics.

The liver-X-receptor (LXR)

LXRs play a key role in regulating the expression of genes involved in cholesterol catabolism, absorption and transport, as well as fatty acid synthesis. Two LXR isoforms are known, i.e., LXRα (NR1H3), that is highly abundant in liver and other tissues involved in lipid metabolism, and LXRβ (NR1H2), that is found in many tissues[85]. Both LXR isoforms bind as heterodimers with the retinoid X receptor (RXR) to specific DNA sequences called LXR response elements (LXRE)[86,87]. LXRs'

natural ligands are oxysterols and 6α-hydroxy bile salts[88,89]. LXR stimulates CYP7A1 transcription (converting cholesterol into bile salts; active in mouse and rats, less active in humans and hamster)[90]. LXR activates SREBP-1c transcription leading to the formation of fatty acids, triglycerides and phospholipids. It thereby induces the synthesis of oleate, which is the preferred fatty acid for the synthesis of cholesteryl esters, which are both necessary for transport and storage of cholesterol[91]. LXR induces the ABC transporters ABCA1, ABCG1, ABCG5, ABCG8, mediating cellular cholesterol efflux[92,93,94]. LXR also induces the transcription of genes encoding apolipoprotein E, CETP and LPL[95,96,97].

The farnesoid-X-receptor (FXR)

FXR (NR1H4) regulates the uptake of bile salts in the enterohepatic circulation and the feedback inhibition of bile salt synthesis by bile salts. FXR acts after heterodimerisation to RXR and is mainly expressed in liver, gut, kidney and adrenal cortex[98,99]. Bile salts are activating ligands of FXR. Activation of FXR by bile salts inhibits transcription of, amongst others, genes encoding *Cyp7A1* and *Cyp8B1*[100,101,102]. This effect of FXR is indirect: activated FXR stimulates transcription of the small heterodimer partner (SHP). SHP subsequently forms a heterodimer with the liver receptor homologue (LRH-1). The SHP / LRH-1 complex dissociates from the promoters of *Cyp7A1* and *Cyp8B1*, which decrease transcription of these genes[100,101,102]. FXR also inhibits NTCP gene transcription via SHP[103]. FXR activates genes encoding IBABP, Abcb11, Abcc2, PLTP and ApoCII[104-108].

The peroxisome proliferator-activated receptors (PPAR)

PPARs play a central role in regulating the storage and catabolism of dietary fats. There are three PPAR subtypes, PPARα (NR1C1), PPARγ (NR1C3) and PPARδ (NR1C2, also called PPARβ). PPARs are activated by fatty acids, eicosanoids (derived from archidonic acid) and a variety of synthetic ligands. PPARS bind as heterodimers with RXR to peroxisome proliferator response elements (PPRE) in the target gene (see Willson TM for review[109]).

PPARα suppresses *Cyp7A1* mRNA levels by reducing the availability of HNF-4α for binding to the *Cyp7A1* promoter and thereby interfering with HNF-4α activation of *CYP7A1*[110]. PPARα stimulates CYP8B1 activity and increases cholic acid synthesis[111]. Binding to PPARα by fatty acid, eicosanoid and fibrate drug ligands leads to activation of numerous genes involved in cellular uptake of fatty acids (fatty acid transport protein) and their initial derivatization for entry in the β-oxidation pathway (acyl-CoA synthetase)[112]. Increased diversion of fatty acids into β-oxidation decreases the availability of fatty-acyl CoA substrates for triglyceride synthesis and, therefore, VLDL formation. PPARα inhibits expression of apoCIII and increases the expression of LPL[113,114]. PPARα and PPARγ activate ABCA1 expression in macrophages via enhanced transcription of LXRα[115]. PPARδ

agonists also enhance expression of ABCA1[116]. Both PPARα and PPARγ ligands increase SR-B1 expression in cultured macrophages[117].

PPARγ stimulates adipogenesis, increases the expression of genes promoting fatty acid storage in adipocytes (fatty-acid binding protein, LPL, acyl-CoA synthase) and represses genes that induce lipolysis and release of fatty acids (see for review[118,119]). PPARγ expression is stimulated by insulin[120]. PPARγ-mediated induction of genes favoring lipid storage in adipose tissue promotes fatty acid repartitioning towards fat rather than muscle, which increases glucose metabolism in muscle[121]. PPARγ induces the expression of genes involved in glucose uptake (Glut 4)[122], and insulin signalling (IRS-1, IRS-2, PI_3-kinase) in adipose tissue and muscle[123].

Hepatocyte nuclear factor 4α (HNF4α)

HNF4α regulates genes involved in lipid metabolism and transport (including apolipoproteins and microsomal triglyceride transfer protein) and glucose metabolism[124,125,126,127,128]. HNF4α stimulates Cyp7A1 gene expression[129,130,131,132].

Liver receptor homologue (LRH-1)

LRH-1 (α-Fetoprotein transcription factor, NR5A2) may be involved in feedback inhibition of bile salt synthesis and in stimulation of bile salt and sterol transport and intestinal absorption. LRH-1 inhibits rat CYP7A1 and human CYP8B1 in response to bile salts[131,132,133]. LRH-1 induces Abcc3 (multidrug-resistant protein-3 or MRP3) gene involved in excretion of bile salts across the basolateral membrane into portal blood[134,135]. Recently, LRH-1 has been shown to act as a transcription factor promoting ABCG5 and ABCG8 expression, thereby inducing hepato-biliary cholesterol excretion and reducing intestinal cholesterol absorption[136].

AIM OF THE THESIS

Specific alterations in triglyceride and cholesterol metabolism may contribute to the development of atherosclerosis in type 1 diabetes. The scope of this thesis was to delineate the alterations in cholesterol and bile salt metabolism that occur in association with type 1 diabetes, with particular emphasis on transporters in liver and intestine. The studies were performed in children and late teenagers with type 1 diabetes and in rats with streptozotocin-induced diabetes. As described earlier, elevated concentrations of both cholesterol and triglycerides have been described in children with diabetes type 1 in poor metabolic control. These lipid concentrations tend to decrease to normal values upon improvement of metabolic control[53,54,137]. In adults with cardiovascular disease and with type 2 diabetes or type 1 diabetes, a delayed chylomicron clearance rate has been described that may contribute to the development of atherosclerosis[24,138,139]. No studies on chylomicron clearance have been reported so far in children and adolescents with type 1 diabetes. In

chapter 2 we investigated whether a delayed clearance rate of chylomicrons may contribute to higher lipid concentrations, especially triglycerides, in late teenagers with type 1 diabetes. We also determined whether CM clearance was correlated with the level of metabolic control, as characterized by HbA_{1c}.

Studies in adult patients with type 1 diabetes showed increased cholesterol absorption and decreased cholesterol synthesis compared to non-diabetic controls and especially to adults with type 2 diabetes[140,141]. In **chapter 3** cholesterol absorption and cholesterol synthesis were estimated in children and late teenagers with type 1 diabetes by measuring total serum values of noncholesterol sterols as markers. The sterol concentrations were related to metabolic control (HbA_{1c}), BMI, insulin dosage and age.

The ABC transporters Abcg5 and Abcg8 are involved in hepato-biliary and intestinal cholesterol transport. In humans, mutations in the genes encoding ABCG5 and ABCG8 are associated with sitosterolemia. In sitosterolemia, biliary excretion of cholesterol is decreased and intestinal absorption of plant sterols and cholesterol is increased[142,143,144]. In Abcg5/Abcg8-deficient mice biliary excretion of cholesterol is also decreased[68]. Overexpression of human ABCG5 and ABCG8 in transgenic mice[66] and pharmacological induction of the transporters in wild-type mice[145] stimulated biliary cholesterol secretion and reduced intestinal cholesterol absorption. In **chapter 4**, an evaluation of the half-transporters Abcg5 and Abcg8 in rats with streptozotocin induced diabetes is described, testing the hypothesis that the increased intestinal cholesterol absorption and decreased hepato-biliary cholesterol excretion in type 1 diabetes is related to a lower expression of Abcg5 and Abcg8 in intestine and liver.

The formation of bile is altered in humans and experimental animals with diabetes mellitus[146-149]. Increased biliary bile salt and phospholipid output rates have been described in rats with streptozotocin (STZ)- or alloxan-induced diabetes[148,149,150]. The secretory rate maximum (SRm) of bile salts, as determined during intravenous bile salt infusions at supraphysiological rates, seemed to be higher in alloxan-induced diabetic rats than in controls[150]. In contrast, biliary glutathione secretion was decreased in diabetic rats[151]. As described earlier, several ABC transport proteins involved in hepato-biliary transport of bile constituents have been identified[152]. In **chapter 5**, we aimed to elucidate whether STZ-induced diabetes affects the hepatic expression of the ABC transporters Abcb4, Abcb11 and Abcc2, and whether such effects would coincide with changes in bile formation in the diabetic state.

In diabetes mellitus an enlarged bile salt pool size has been described in humans and experimental animals[146,148]. In experimental diabetic animals, an association with an increased bile salt synthesis has been found[153]. In **chapter 6** cholate pool size and synthesis rate were determined with a relatively new isotope dilution technique using $^2H_4$-cholate in unanaesthesized STZ-diabetic, control and

insulin treated STZ-diabetic rats. An estimation of bile salt absorption in diabetes compared to controls and insulin treatment was calculated. Kinetic parameters were related to the expression of relevant genes in bile salt synthesis and involved hepatic transcription factors, especially the bile salt- activated nuclear receptor FXR. Bile salt absorption was correlated to the expression of intestinal transport proteins involved in intestinal bile salt transport.

In **chapter 7** the significance of the data presented, is discussed and recommendations are made for future research.

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2 Postprandial chylomicron clearance rate in late teenagers with diabetes mellitus type 1

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ABSTRACT

Background/Aims: A delayed chylomicron (CM) clearance rate, a known risk factor for atherosclerosis, has been described in adults with diabetes type 1 (DM1). We determined the CM clearance rate in late teenagers with DM1, and the relationship between CM clearance rate and elevated plasma lipid concentrations in DM1 teenagers in poor metabolic control (as characterized by HbA_{1c} percentage).

Methods: Plasma lipids and CM clearance were determined in 9 patients with DM1 (mean age ± SD: 17.5 ± 0.6 y) and 4 healthy controls (mean age ± SD: 20.1 ± 0.8 y), by measuring breath $^{13}CO_2$, and plasma triglyceride, retinyl palmitate and ^{13}C-labeled oleic acid concentrations, after oral administration of a fat-rich meal together with vitamin A and ^{13}C-oleic acid.

Results: In patients with DM1 fasting triglyceride and cholesterol concentrations were positively correlated with HbA_{1c} percentage ($p<0.05$). Neither in DM1 patients nor in controls, was an elevated triglyceride concentration (above 1.7 mmol/l) found. Yet, in 22 % of DM1 patients, cholesterol concentration was above 5.2 mmol/l, but not in any of the controls. CM clearance rate in DM1 patients was similar to that in controls, and did not significantly correlate with HbA_{1c} percentage. Fasting lipid concentrations in DM1 patients were not significantly correlated with CM clearance rate.

Conclusions: Present data indicate that elevated lipid concentrations in late teenagers with DM1 are not attributable to a delay in CM clearance rate. A delayed CM clearance rate at late teenager age is not a risk factor contributing to the increased risk for atherosclerosis in DM1.

INTRODUCTION

Diabetes mellitus type 1 (DM1) is associated with a 2-4 fold increased risk for cardiovascular disease[1]. Specific diabetes-related phenomena have been suggested to play a role in the observed association, such as glycosylation of proteins[2], presence of renal disease[3], elevated concentrations of plasma cholesterol, triglycerides and low density lipoprotein (LDL) cholesterol[4], and altered composition of very low density lipoprotein (VLDL) and LDL[5,6]. In children and late teenagers, studies on risk factors associated with DM1 and atherosclerosis have mainly focused on cholesterol and triglyceride concentrations[7-9]. Elevated concentrations of both cholesterol and triglycerides have been reported in children with DM1 in poor metabolic control, which tended to decrease to non-diabetic values upon improvement of metabolic control[7-9].

A delayed chylomicron (CM) clearance has been identified as a risk factor for atherosclerosis in patients with coronary heart disease, in patients with type 2 diabetes and in *adult* patients with DM1[10-12]. Insulin enhances the metabolism of CM, by stimulating the hydrolysis of CM triglycerides by lipoprotein lipase, and the hepatic uptake of CM remnants[11]. A relative deficiency of insulin, such as present in DM1, could therefore lead to a delay in CM clearance. A delay in CM clearance would imply a prolonged postprandial hyperlipidemia, which supposedly leads to increased deposition of CM contents into the arterial wall, and to an unfavorable translocation of cholesteryl-esters from HDL to CM[13,14]. Delays in CM clearance are amenable to dietary intervention, by changing the intake of fish oil (long chain PUFA)[15], monounsaturated-fat[16], or carbohydrate[17]. Yet, it is not known whether CM clearance is delayed in *late teenagers* with DM1. In non-diabetic individuals CM clearance rate appeared to be inversely correlated with age (i.e. a delayed clearance in older individuals)[18,19]. Specific dietary recommendations to late teenagers with DM1 are only warranted if CM clearance would be delayed at their age. In the present study, we investigated, whether a delayed clearance rate of chylomicrons (CM) can be a risk factor for atherosclerosis and may contribute to higher lipid concentrations, especially triglycerides, in late teenagers with DM1. We also determined whether CM clearance is correlated with the level of metabolic control, as characterized by HbA_{1c}. CM clearance was investigated by determination of plasma concentrations of three CM constituents (triglycerides, vitamin A and ^{13}C-oleic acid), after their prior oral ingestion.

PATIENTS AND METHODS

Subjects

Chylomicron clearance was studied in nine late teenagers with DM1 (four boys, five girls; mean age ± SD: 17.5 ± 0.6 y) and four healthy controls (two boys, two

girls; mean age ± SD: 20.1 ± 0.8 y). Patients were studied at late teenager age to rule out the effect of puberty on lipid metabolism, as puberty is associated with a relative insulin resistance[20]. DM1 patients were selected from our outpatient clinic population with fair or poor metabolic control (5 with HbA_{1c} < 8.7%, 4 with HbA_{1c} > 9.5%). Patients were selected on basis of their HbA_{1c} (< 8.7%. > 9.5%), and differed only in metabolic control. HbA_{1c} ± SD at the test day were 7.8 ± 0.5% and 10.9 ± 1.5%, respectively. Mean HbA_{1c} in the year preceding the test were 7.6 ± 0.4% and 11.2 ± 1.9 %, respectively. Patients with microvascular complications like microalbuminuria (defined as albumin excretion rate >30 mg/24 h in 24 h urine collection), or retinopathy (ophthalmoscopy through dilated pupils by ophthalmologist) were excluded from the study. Other exclusion criteria were severe insulin resistance (insulin dosages above 1.5 U/kg/day), with diabetes associated diseases like hypothyroidism and celiac disease, renal-, or liver disease, obesity (weight for height > 90th percentile), anorexia nervosa (weight for height < 10th percentile), first grade relatives with lipid disturbances, or cardiovascular disease under the age of 60. Patients, who had had diabetes for less than one year were also excluded. No medication known to affect lipoprotein metabolism was used by the subjects, except for oral contraceptives (3 of DM1, 2 of controls) and insulin. In control subjects, the absence of microalbuminuria was tested. They had none of the above mentioned diseases. At palpation control subjects did not have a goiter, and they had no known first grade relatives with lipid disturbances or cardiovascular disease under the age of 60. Mean insulin dosage was not significantly different between patients with HbA_{1c} below 8.7% or above 9.5% (0.9 ± 0.2 U/kg/day in each group). Patients with DM1 did not have ketones in their urine, nor any sign of illness during the three days preceding the test. Informed consent was obtained from all patients and controls over 18 years of age. Parental informed consent was obtained for minors. The study was approved by the medical ethical committee of our hospital.

Study design

In DM1 patients and controls, CM metabolism was investigated after an overnight fast. CM metabolism was investigated by analyzing plasma disappearance of 3 CM constituents. First, the disappearance rate of the main CM core component, triglycerides, was measured after ingestion of a high-fat meal. Second, a classic test to quantitate uptake of CM remnants by the liver, the vitamin A test, was applied[21,22]. After oral ingestion, vitamin A is taken up by enterocytes and incorporated into the core of CM in the form of retinyl esters. Retinyl esters remain associated with the CM in the circulation until the remnant stage, after which they are taken up by the liver[21]. Third, hydrolysis of CM triglycerides by lipoprotein lipase was investigated by ingestion of a stable isotopically labeled fatty acid (^{13}C-oleic acid). Orally ingested ^{13}C labeled fatty acid will be incorporated in CM triglycerides. After

appearance in plasma, ^{13}C-triglycerides will be hydrolyzed by lipoprotein lipase and the hydrolyzed ^{13}C-fatty acid will be taken up into body cells and metabolized. The metabolism of ^{13}C labeled CM fatty acids can be quantified by measuring the disappearance of ^{13}C labeled fatty acid from plasma and, indirectly, by determining the appearance of $^{13}CO_2$ in breath, reflecting oxidation of the parent compound. Subjects were instructed to avoid food naturally enriched in ^{13}C, such as corn, pine apple, corn-flour, cane-sugar, millet for three days before the test.

After an overnight fast, a standardized high-fat meal (composition: 1 g fat/kg body weight (BW), 60% (wt/wt) saturated fat; 1 g carbohydrate/kg BW, 0.5 g protein/kg BW), together with vitamin A (50.000 IU/m^2) and [1- ^{13}C]-oleic acid (5 mg/kg BW) was ingested between 8.30 and 9.00 AM (time 0). The high-fat meal consisted of a milkshake and wheat bread with butter and cheese. For 6 h after ingestion of the testmeal, no other food was ingested. DM1 patients administered their insulin in an adjusted dosage 30 minutes before the testmeal. The dosage of short acting insulin was adjusted for the carbohydrate composition in the testmeal (compared to their normal carbohydrate intake at breakfast and at 10.30 AM) and the glucose value at 8.00 AM. The dosage of long acting insulin was lowered in view of the fasting period of 6 h after the testmeal. Before and for 6 hours after ingestion of the testmeal, every half hour breath samples were collected to quantitate breath $^{13}CO_2$, and every hour blood samples were collected to quantitate plasma concentrations of retinyl palmitate, triglycerides, cholesterol, ^{13}C-oleic acid, glucose and FFA. In the first blood sample taken, HbA_{1c} concentration was determined.

Analytical methods

Glucose levels were measured on a calibrated Companion 2 glucosemeter (Medisense, Abbott laboratories, Abbott Park, IL). Triglyceride and cholesterol concentrations were determined in plasma by enzymatic methods using commercial kits (Triglycerides/GB and Chol respectively, Boehringer, Mannheim, Germany), as were FFA in plasma (NEFA C, Wako, Neuss, Germany). Plasma retinyl palmitate concentrations were determined by high pressure liquid chromatography (HPLC), according to Bui and to Zaman et al (23,24). HbA_{1c} was determined by ion-exchange HPLC (VARIANT™ HbA_{1c} Program with Bio-Rad VARIANT Hemoglobin Testing System, Bio-Rad, Hercules, CA). Normal range of HbA_{1c} in non-diabetic individuals in our hospital is 4.6-6.1%.

^{13}C-oleic acid content was measured in total plasma lipid. To determine ^{13}C-oleic acid, plasma lipids were methylated and extracted according to Lepage and Roy[25]. Resulting fatty acid methyl esters were analyzed by gas chromatography (GC) to quantitate oleic acid concentration[26] and by GC combustion isotope ratio mass spectrometry (GC-C-IRMS) to determine ^{13}C-enrichment of oleic acid. Fatty acids were quantified using heptadecanoic acid (17:0) as internal

standard. ^{13}C-enrichment of the oleic acid methyl esters was determined by using a Finnigan MAT Delta S isotope ratio mass spectrometer interfaced to a Varian 3400 gas chromatograph via a capillary oxidation furnace (Finnigan MAT, Bremen, Germany). Separation of the methyl esters and determination of [^{13}C] abundance was performed according to the method described by us previously[27]. The concentration of ^{13}C-oleic acid in plasma is expressed as the percentage of the dose administered per liter plasma (% Dose/L).

^{13}C-enrichment in the breath samples was determined by means of continuous flow isotope ratio mass spectrometry (Finnigan Breath MAT, Finnigan MAT Gmb, Bremen, Germany). The ^{13}C abundance of breath CO_2 was expressed as the difference (‰) compared with the reference standard Pee Dee Belemnite limestone ($\delta\ ^{13}C_{pdb}$, ‰). The proportion of ^{13}C-label excreted in breath CO_2 was expressed as the percentage of administered ^{13}C-label recovered per hour (%^{13}C Dose/h), and was corrected for individual CO_2 production as determined every hour for 5 minutes by indirect calorimetry (Oxycon, model ox-4, Dräger, Breda, The Netherlands).

Statistical analysis

Results are expressed as means ± SD. Group means were compared by Student-t test, or, if SDs were significantly different, by Welch's alternate-t test. The postprandial clearance rates of retinyl palmitate and triglycerides were calculated in individual subjects, by calculating the slope of the linear regression line, using the peak concentration and plasma concentrations up to 3 h after peak concentration as dependent variables, and corresponding time points as independent variables in each individual. The slopes in DM1 and control late teenagers (means ± SD) were compared by Student-t test. The postprandial clearance of retinyl palmitate was also investigated by calculating the area under the retinyl palmitate curves of each individual and comparing the 2 group means and SDs by Student-t test.

RESULTS

Base line characteristics of DM1 patients and controls

Table 1 shows the clinical data and fasting cholesterol and triglyceride concentrations in DM1 patients and controls. For triglyceride concentrations in children and teenagers a cut-off concentration of 1.7 mmol/l is used (as defined by the European Non-Insulin-Dependent Diabetes Policy Group)[7]. In none of the DM1 patients or controls, fasting triglyceride concentrations were higher than 1.7 mmol/l. According to the National Cholesterol Education Program (NCEP), fasting cholesterol concentrations in healthy children and teenagers are classified as "borderline increased", when between 4.4 and 5.2 mmol/l, and as "high", when exceeding 5.2 mmol/l (28). Fasting cholesterol concentrations were higher than

Table 1: Clinical data and fasting lipid concentrations in late teenagers with DM1 and controls.

	DM1 patients (n=9)	Controls (n=4)	Significance
Age (y)	17.6 ± 0.6	20.1 ± 0.8	p < 0.01
Body mass index (kg/m2)	22.3 ± 2.0	22.1 ± 2.3	NS
Duration of diabetes (y)	8.8 ± 3.6		
Insulin dosage (IU/kg BW/day)	0.9 ± 0.2		
HbA1c (%)	9.2 ± 1.9	5.1 ± 0.2	p < 0.01
Plasma cholesterol (mM)	4.0 ± 1.1	4.1 ± 0.5	NS
Plasma triglycerides (mM)	0.7 ± 0.4	0.6 ± 0.3	NS

NS= not significant
Values represent means ± SD

5.2 mmol/l in 22% (2 of 9) of the DM1 patients and none of the controls. Borderline increased cholesterol concentrations were found in 1 of 9 DM1 patients and 1 of 4 controls. DM1 patients and controls had similar body mass indices. As expected, HbA_{1c} percentage was significantly higher in patients with DM1 compared with controls ($p<0.01$). The controls were slightly older than the patients ($p<0.01$). In DM1 patients, fasting triglyceride and cholesterol concentrations were positively correlated to HbA_{1c} percentage ($p<0.05$). Fasting plasma triglyceride concentration was correlated with HbA_{1c}, with the following equation: [fasting plasma triglyceride concentration] = $0.164[HbA_{1c}]-0.794$ ($r=0.80$, $p<0.01$). Fasting plasma cholesterol concentration was correlated with HbA_{1c} with the following equation: [fasting plasma cholesterol concentration] = $0.45[HbA_{1c}]-0.147$ ($r=0.76$, $p<0.05$).

Postprandial response: glucose, free fatty acids and triglycerides.

Fasting and postprandial glucose concentrations were significantly higher in patients with diabetes compared with controls ($p<0.01$, Figure 1A). In patients with diabetes, maximal glucose concentrations were found at 2h after ingestion of the high-fat meal. FFA concentrations were significantly higher in patients with diabetes compared with controls, before (timepoint 0) and at 1 and 2 h after administration of the testmeal ($p<0.01$), but were similar thereafter (Figure 1B).

Postprandial triglyceride concentrations showed maximal values at 2h (DM1) or 3h (controls) after ingestion of the test meal (Figure 2A). Fasting and postprandial triglyceride concentrations were not significantly different between the two groups at any timepoint. Also, the rate of decrease in triglyceride concentration, after reaching its maximum, was not significantly different between the 2 groups, as calculated from individual decay regression lines ($p>0.05$; slope of regression line

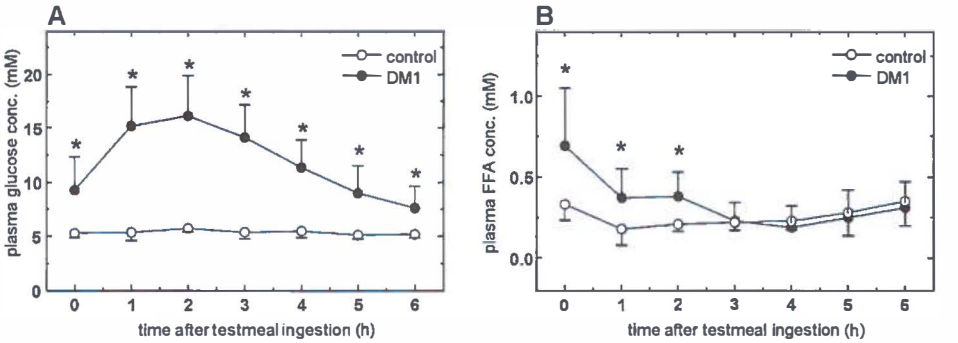

Figure 1: Fasting and postprandial plasma glucose concentrations **(A)** and free fatty acid concentrations **(B)** in late teenagers with diabetes mellitus type 1 (n=9, closed symbols), and non-diabetic controls (n=4, open symbols), before and for 6 h after ingestion of a fat-rich meal at timepoint 0. Insulin dosage was adjusted for the carbohydrate composition of the testmeal, the glucose concentration at the beginning of the test, and the fasting period of 6 h after the testmeal. * $p<0.05$, reflects a significant difference between the two groups.

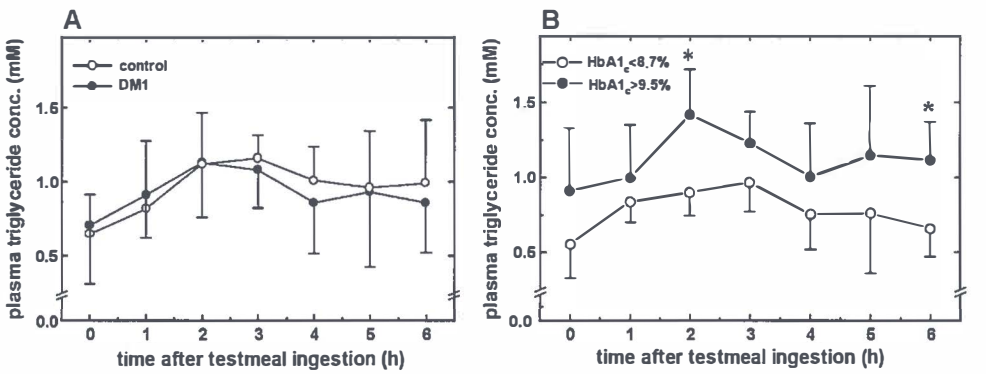

Figure 2: Fasting and postprandial plasma triglyceride concentrations in late teenagers with diabetes mellitus type 1 (n=9, closed symbols), and non-diabetic controls (n=4, open symbols)**(A)** and in DM1 patients with HbA_{1c} > 9.5% (n=4, closed symbols) and HbA_{1c} < 8.7% (n=5, open symbols)**(B)**. A fat-rich meal was ingested at time point 0, afterwards no food was taken for 6 h. Results between the total diabetes and control group were not significantly different at any time point **(A)**. In DM1 patients with HbA_{1c} > 9.5% plasma triglyceride concentrations were significantly higher at time point 2 and 6 h compared to DM1 patients with HbA_{1c} < 8.7% (*$p<0.05$)**(B)**.

in DM1 and controls -0.32 ± 0.19 and -0.14 ± 0.08 mmol L^{-1} h^{-1}, respectively). If postprandial triglyceride concentrations were compared between DM1 patients with high and moderate HbA_{1c} (Figure 2B), the patients with high HbA_{1c} (>9.5%) had significantly higher plasma triglyceride concentrations at timepoint 2 and 6 hours compared to the patients with moderate HbA_{1c} (<8.7%)($p<0.05$). Yet, these significant differences disappeared, when the percentage increase in triglyceride concentration from timepoint 0 was calculated; indicating that higher postprandial triglyceride concentrations in DM1 patients with HbA_{1c} > 9.5% were due to higher fasting triglyceride concentrations.

Vitamin A test

Figure 3 shows that in diabetic and in control late teenagers, mean concentrations of retinyl palmitate were maximal at 2h after the ingestion of the vitamin A-containing high-fat meal. Except for the 6h time point, no significant differences were observed between the 2 groups in plasma concentrations of retinyl palmitate. The areas under the curve were 7.0 ± 3.1 and 8.9 ± 2.9 in DM1 patients and controls, respectively ($p>0.05$). Based on individual measurements, the rates of decrease in retinyl palmitate concentrations, after reaching its maximum, were not significantly different between the 2 groups ($p>0.05$; slope of regression line in DM1 and controls, -0.67 ± 0.45 and -0.75 ± 0.37 $\mu mol\ L^{-1}\ h^{-1}$, respectively).

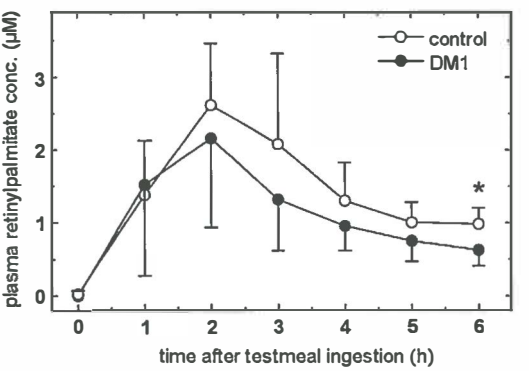

Figure 3: Plasma retinyl palmitate concentrations before and for 6 h after after ingestion of a fat-rich meal and retinyl palmitate, in late teenagers with diabetes mellitus type 1 (n=9, closed symbols), and non-diabetic controls (n=4, open symbols). *$p<0.05$, reflects a significant difference between the two groups.

^{13}C-oleic acid test

Whereas the vitamin A test mainly reflects the uptake of CM remnants in the liver, the ^{13}C-oleic acid test provides information, at least theoretically, on the lipolysis of CM-triglycerides. In case of a decreased activity of lipoprotein lipase during insulin deficiency, one would expect increased plasma^{13}C-oleic acid concentrations, a delayed disappearance of ^{13}C-oleic acid from plasma, and a delayed appearance of $^{13}CO_2$ in breath. However, plasma ^{13}C-oleic acid concentrations were not significantly different between DM1 and control late teenagers after ingestion of ^{13}C-oleic acid together with the high-fat meal ($p>0.05$, Figure 4A). Unlike retinyl palmitate concentrations (Figure 3), plasma ^{13}C-oleic acid concentrations did not reach maximal values at time point 2h, but rather continuously increased throughout the 6h study period.

Detection of $^{13}CO_2$ in breath samples implies oxidation of the administered ^{13}C-oleic acid, indicating lipolysis of CM-triglycerides and cellular uptake of ^{13}C-free fatty acids. Increase in breath $^{13}CO_2$ concentrations was not delayed in DM1

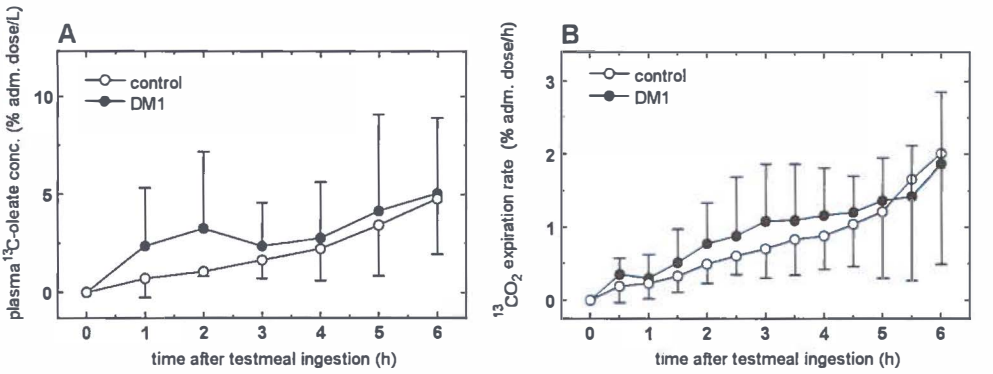

Figure 4 (A) Plasma ^{13}C-oleic acid concentrations before and for 6 h after ingestion of ^{13}C-oleic acid and a fat-rich meal in late teenagers with diabetes mellitus type 1 (n=9, closed symbols), and non-diabetic controls (n=4, open symbols). Results between the 2 groups were not significantly different at any time point. **(B)** Breath $^{13}CO_2$ concentration before and for 6 h after ingestion of ^{13}C-oleic acid and a fat-rich meal in late teenagers with diabetes mellitus type 1 (n=9, closed symbols), and non-diabetic controls (n=4, open symbols). Results between the 2 groups were not significantly different at any time point.

patients, and breath $^{13}CO_2$ concentrations were not significantly different between the 2 groups at any time point (Figure 4B). Breath $^{13}CO_2$- and plasma ^{13}C-oleic acid concentrations appeared significantly correlated ($r=0.93$, $p<0.01$; data not shown).

Chylomicron clearance rate related to HbA_{1c}, gender and fasting triglycerides

CM clearance in DM1 patients in poor metabolic control (arbitrarily chosen at HbA_{1c} > 9.5%) was not delayed in any of the 3 tests (triglycerides, vitamin A, or ^{13}C-oleic acid), compared either with DM1 patients with moderate good metabolic control (HbA_{1c} < 8.7%), or with non-diabetic controls (data not shown). When the slope of the regression lines of triglyceride clearance and of retinyl palmitate clearance were plotted against HbA_{1c}, no significant correlation was found ($p>0.05$; $r=0.10$, $r=0.12$, respectively). Also, the areas under the retinyl palmitate curves were not significantly correlated with HbA_{1c} ($r = 0.22$, $p>0.05$). There was no significant difference between boys or girls with DM1 in CM clearance rates for any of the three tests applied and no significant correlation was found between CM clearance rates and fasting triglyceride concentrations.

DISCUSSION

In the present study we aimed to identify if a delayed CM clearance rate, as a known risk factor for atherosclerosis, is present in late teenagers with DM1, and if a

delayed CM clearance could contribute to the higher plasma lipid concentrations in DM1 teenagers in poor control. In DM1 patients, fasting triglyceride and cholesterol concentrations were positively correlated with the level of metabolic control, as characterized by HbA_{1c}. Our results show that the clearance rate of CM was not delayed in late teenagers with DM1 compared with controls. CM clearance rate was not related with either metabolic control (HbA_{1c}), gender, fasting cholesterol or triglyceride concentrations in late teenagers with DM1, indicating that relatively higher triglyceride and cholesterol concentrations in late teenagers with DM1 cannot be attributed to a delayed clearance of chylomicrons.

The observed correlation between HbA_{1c} and high plasma triglyceride and cholesterol concentrations, is in agreement with other studies in children and late teenagers with DM1[7-9,29]. The pathophysiological mechanisms of elevated lipid concentrations in diabetes type 1 are not fully understood. It has been hypothesized that lipid absorption from the intestine is increased in DM1. Studies in streptozotocin-induced diabetic rats showed an increased intestinal triglyceride production[30] and cholesterol synthesis[31], and an increased intestinal absorption of cholesterol[32]. Another possible mechanism is an increased hepatic VLDL production, as a consequence of increased free-fatty acid release from adipose tissue and as a consequence of inefficient suppresion of hepatic VLDL release by insulin. In poorly controlled adult patients with DM1 the antilipolytic effect of insulin is diminished, leading to a higher free-fatty acid flux from adipose tissue[33]. Hepatic VLDL release in patients with type 2 diabetes was less inhibited by insulin compared with control subjects[34]. Finally, a delayed metabolism and clearance of lipoproteins could lead to hypertriglyceridemia and hypercholesterolemia. A delayed chylomicron and VLDL clearance has been identified in patients with type 2 diabetes and in *adult* patients with DM1[11,12] and this could play a role in hypertriglyceridemia. Increased production of LDL from elevated VLDL, and delayed clearance of (glycosylated) LDL could lead to hypercholesterolemia[35,36]. A reduced hepatic uptake of LDL particles from patients with type 2 diabetes was associated with an altered lipid composition of the LDL particle and glycation of LDL protein[36]. Insulin stimulates the hydrolysis of CM and VLDL triglycerides by lipoprotein lipase[37] and the hepatic uptake of chylomicron-, and VLDL-remnants and LDL in the liver, probably by stimulating the LDL receptor[38,39].

In the present study we investigated the possibility that a delayed CM clearance rate is a possible contributor to the higher lipid values in diabetes in poor control. CM metabolism was studied by analyzing plasma disappearance of 3 different CM constituents. None of the three tests applied indicated significant differences in CM clearance between late teenagers with diabetes and controls, nor between DM1 boys or girls, nor between fairly or poorly controlled DM1 patients. Present data indicates that in patients with DM1, at this late teenager age, a delayed CM clearance rate is not an important atherosclerotic risk factor

and does not contribute to the relatively higher triglyceride concentrations in late teenagers with DM1 in poor metabolic control. It therefore remains to be determined, whether the observed correlation between metabolic control and triglyceride concentrations in late teenagers with diabetes is caused by one of the other hypothesized mechanisms, like increased hepatic VLDL production.

Several confounders could have influenced the outcome of the study. The relatively small study group could have confounded the results, however, since none of the three tests showed a trend towards delayed CM metabolism in DM1 patients, this possibility seems less likely. Due to the relatively small numbers of patients studied, we can not exclude the possibility, that a small difference in CM clearance exists, yet the clinical consequence of such a finding would then be questionable. Remarkably, studies performed in adult patients with type 1 or type 2 diabetes, who did show a difference in CM clearance rates were based on numbers of patients studied comparable to the present study[12,40].

It can be excluded that an acutely deteriorated metabolic control during the test confounded the results. The patients with diabetes did express a relative insulin deficiency compared with the non-diabetic controls, because fasting and postprandial glucose concentrations were increased in the former (Figure 1A). Postprandial FFA concentrations decreased in both groups and were not significantly different between both groups from timepoint 3 h after ingestion of the fat-rich meal, indicating that during the testmeal, insulin levels were sufficient to inhibit lipolysis. It could be possible that the metabolic control of the DM1 patients was better during the test day compared to their daily practice, because of regular glucose control and adapted insulin concentrations. However, insulin concentrations were calculated according to the dosages the patients normally injected themselves, and this adaptation of insulin dosage (based on the normally injected insulin dosage) was comparable with other studies[12,41]. It is therefore unlikely that "over-regulation" has confounded our present study. Finally, postprandial lipoprotein metabolism is highly dependent on fasting plasma triglyceride concentration[42], and in the presented study group none of the subjects had fasting triglyceride concentrations > 1.7 mmol/l. However, studies in normotriglyceridemic patients with type 2 diabetes and adults with DM1 did show a delayed post-prandial chylomicron clearance[12,43].

The presented results indicate that lipoprotein lipase activity and uptake of CM remnants in the liver are sufficiently stimulated in late teenagers in the diabetic state. The observed difference between the present results in late teenagers with DM1 (no delayed CM clearance rate) and previous data in adults with DM1 (delayed CM clearance rate) could be due to a worse metabolic regulation. Georgopoulos and Phair studied only adults with DM1 in poor control (HbA_1 12.8 ± 0.6%; normal range: 3.9-7.7%)[12], whereas in our study DM1 patients were in fair or in poor control (mean HbA_{1c} 9.2 ± 1.9%; normal range: 4.6-6.1%). Comparison of

metabolic regulation (HbA_{1c}) between the 2 studies is difficult however, because in the first study HbA_1 percentages were presented instead of HbA_{1c}, and HbA_1 was measured by agarose gel electrophoresis rather than by HPLC[44]. Another explanation for the difference in CM clearance rate could be the age dependency of CM clearance. Studies in healthy individuals using the vitamin A test have shown that CM clearance rate delays with increasing age[18]. It is tempting to speculate that DM1 patients do have an age dependency in CM clearance, similarly to subjects without DM1.However, the age-dependent delay in CM clearance rate is more pronounced in the former.

In conclusion, the present study showed that higher lipid concentrations in late teenagers with DM1 in poor control, were not caused by a delay in CM clearance rate. Present data indicate that a delayed CM clearance rate at late teenager age is not a risk factor contributing to the increased risk for atherosclerosis in DM1.

Acknowledgements

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3 Decreased cholesterol synthesis in children and adolescents with type 1 diabetes mellitus

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Submitted

ABSTRACT

Background/Aims: Type 1 diabetes mellitus (DM1) is, in adults, associated with increased intestinal cholesterol absorption and decreased cholesterol synthesis. No information is available on cholesterol absorption and synthesis in children with DM1. Serum levels of plantsterols and intermediates in cholesterol biosynthesis provide surrogate markers for these processes.

Methods: Non-fasting serum lipid concentrations and indices for cholesterol absorption (i.e. serum concentrations of cholestanol, campesterol and sitosterol) and for cholesterol synthesis (serum lathosterol concentration) were evaluated in 65 children with DM1 (mean age 11.0 ± 3.7 y) and 17 controls (mean age 12.4 ± 4.6 y).

Results: Total cholesterol and LDL cholesterol concentrations were significantly higher in female, but not in male patients with DM1 compared with controls. In patients with DM1, cholesterol and LDL cholesterol concentrations were positively correlated with HbA_{1c} ($p < 0.01$). LDL cholesterol and cholesterol/HDL cholesterol ratio in DM1 patients positively correlated with age. The lathosterol/cholesterol ratio in DM1 patients was significantly lower compared with controls (-38%, $p < 0.01$). Indices for cholesterol absorption were increased in absolute concentrations (+23-35%, $p < 0.05$) in DM1 patients, but were unaffected when expressed relative to cholesterol concentrations. In DM1 patients and controls, cholesterol synthesis was negatively correlated with cholesterol absorption ($r = -0.43$, $p < 0.01$). In DM1 patients and controls, cholesterol absorption decreased with age, whereas cholesterol synthesis increased with age (in DM1, $r = -0.27$, $p < 0.05$; $r = 0.25$, $p < 0.05$, respectively; in controls, $r = -0.51$, $p < 0.05$; $r = 0.29$, $p > 0.05$, respectively).

Conclusions: Our data indicate that children with DM1 have a decreased rate of cholesterol synthesis, which seems partly attributable to increased cholesterol absorption.

INTRODUCTION

Type 1 diabetes mellitus (DM1) is associated with an elevated risk for atherosclerosis. In the Framingham study, patients with type 1 diabetes older than 55 years had a four times increased mortality risk caused by coronary artery disease compared to controls[1]. Factors contributing to the increased atherosclerotic risk in DM1 are elevated serum triglycerides and LDL cholesterol and decreased HDL cholesterol levels[2,3,4].

LDL cholesterol in DM1 can be elevated by a variety of factors. In rats with streptozotocin (STZ)-induced diabetes, an increased intestinal absorption of cholesterol associated with a decreased hepatic cholesterol synthesis and suppressed hepatic LDL receptor expression has been found[5], suggesting that hyperabsorption of (dietary) cholesterol leads to delayed LDL clearance. The enhanced intestinal cholesterol absorption in STZ-rats has been associated with hyperphagia[5,6], with morphological changes of the rat intestine[7,8,9] and, more recently, with decreased expression of the ATP-Binding Cassette half-transporters Abcg5 and Abcg8[10]. A decreased activity of Abcg5 and Abcg8 transport proteins in enterocytes limits the cholesterol transport from the enterocyte back into the intestinal lumen, resulting in increased net cholesterol uptake[11,12,13].

Only few studies addressing cholesterol absorption have been performed in human subjects with type 1 diabetes. Serum campesterol/cholesterol ratios and sitosterol/cholesterol ratios as markers of cholesterol absorption were increased in adult DM1 patients in poor glycemic control when compared with controls and with DM1 patients on intensive insulin therapy[14]. Markers of cholesterol absorption were increased and those of cholesterol synthesis decreased in adult patients wit DM1 in good glycemic control compared with adult patients with type 2 diabetes and with control subjects[15,16]. To our knowledge, no studies have been performed on cholesterol absorption and synthesis in children and adolescents with type 1 diabetes. Since the formation of atherosclerotic plaques may initiate already at a young age, it is of importance to gain insight in potential disturbances in cholesterol metabolism in children with DM1 to provide a background for early interventions in this specific population aimed at prevention of atherosclerosis later in life. Furthermore, we showed previously that specific changes in lipid metabolism in DM1 described in adults, such as delayed postprandial chylomicron clearance, are not present in children with DM1[17].

The aim of the present study was to evaluate cholesterol absorption and cholesterol synthesis in children and adolescents with DM1 in comparison with children and adolescents without this disease. Non-fasting serum lathosterol/cholesterol ratio was used as a marker for cholesterol synthesis and cholestanol/cholesterol, campesterol/cholesterol and sitosterol/cholesterol ratios as markers for cholesterol absorption. Serum sterol/cholesterol ratios were correlated with glycemic control (HbA_{1c}), insulin dosage, BMI, gender and age. Our data indicate

that cholesterol synthesis is reduced in children with DM1, possibly related to the rate of intestinal cholesterol absorption.

RESEARCH DESIGN AND METHODS

Subjects

From our diabetic outpatient clinic, 30 boys and 35 girls, mean age 11.0 ± 3.7 years (mean ± SD) were included. Mean BMI standard deviation score for age was 0.35 ± 0.84 SD (mean ± SD), HbA_{1c} 8.2 ± 1.0 %, mean insulin dosage per kilogram body weight 0.95 ± 0.25 U/kg/day and mean duration of diabetes was 5.1 ± 2.9 years (Table 1). Control subjects were obtained from our endocrine outpatient clinic and consisted of patients with familial tall stature (n = 7), congenital hypothyroidism well supplemented with thyroid hormone (n = 4), delayed puberty (n=3), Graves disease treated with radioactive iodide and well supplemented with thyroid hormone (n=1), multiple endocrine neoplasia syndrome type 2A in good control (n=1) and idiopatic short stature (n=1). The control group consisted of 8 boys and 9 girls, mean age 12.4 ± 4.6 years, with a BMI standard deviation score for age of mean 0.21 ± 1.52 SD (Table 1). Five DM1 patients had microalbuminuria (defined as albumin excretion rate > 30 mg/24 h in 24 h urine collection), no patients had retinopathy (ophthalmoscopy through dilated pupils by ophthalmologist). No patients had severe insulin resistance (insulin dosages above 1.5 U/kg/day). One patient with DM1 had also hypothyroidism, well regulated with medication, and one other patient with DM1 had also celiac disease, and used a gluten-free diet. None of the patients or controls used spreads containing plantsterols in their regular diet.

Non-fasted blood samples were taken during the daytime when a blood sample had to be taken for a regular metabolic control. Informed consent was obtained from all patients and controls or, if applicable, from their parents. Both patients and controls were asked if they used spreads with extra plant sterols. Body mass index standard deviation scores for age were calculated using the Dutch reference data from 1997[18].

Analytical methods

Plasma concentrations of total cholesterol and triglycerides were measured with commercially available kits (Merck, Maria Stein, OH). HDL cholesterol was measured using a commercially available assay system (MERCK MEGA (Merck KGaA, Darmstadt, Germany) with EZ HDL cholesterol reagent (Trinity/Biotech, Jamestown, NY). LDL cholesterol was calculated with the Friedewald formula. HbA_{1c} was determined by ion exchange HPLC (VARIANT™ HbA_{1c} Program with Bio-Rad VARIANT Hb Testing system, Bio-Rad, Hercules, CA). Normal range of HbA_{1c} in individuals without diabetes is 4.2-6.1%. Serum lathosterol, cholestanol,

Table 1. Clinical data and serum lipids in children with type 1 diabetes and controls

	Patients with type 1 diabetes n = 65	Controls n = 17
Age (y)	11.0 ± 3.7	12.4 ± 4.6
Gender	35 female / 30 male (54% female)	9 female / 8 male (53% female)
Body mass index (SD for age)	0.35 ± 0.84	0.21 ± 1.53
Duration of diabetes (y)	5.12 ± 2.89	n.a.
Insulin dose (U/kg/day)	0.95 ± 0.25	n.a.
HbA1c (%)	8.15 ± 1.04	not investigated
Triglycerides (mM)	1.03 ± 0.56	1.23 ± 0.47
Male	0.97 ± 0.52	1.27 ± 0.52
Female	1.08 ± 0.59	1.20 ± 0.44
Cholesterol (mM)	4.29 ± 0.84	4.06 ± 0.76
Male	4.02 ± 0.81	4.40 ± 0.75
Female	4.52 ± 0.81[ab]	3.76 ± 0.68
LDL cholesterol (mM)	2.43 ± 0.71	2.19 ± 0.53
Male	2.26 ± 0.68	2.49 ± 0.41
Female	2.56 ± 0.71[a]	1.93 ± 0.49[c]
HDL cholesterol (mM)	1.39 ± 0.37	1.31 ± 0.41
Male	1.31 ± 0.37	1.33 ± 0.53
Female	1.47 ± 0.36	1.29 ± 0.29
Ratio cholesterol/HDL cholesterol	3.22 ± 0.82	3.27 ± 0.87
Male	3.24 ± 0.97	3.61 ± 1.12
Female	3.19 ± 0.68	2.96 ± 0.44

Values represent means ± SD. a: $p < 0.05$ compared to controls, b: $p < 0.05$ compared to male patients, c: $p < 0.05$ compared to male controls, n.a.: not applicable

campesterol and sitosterol concentrations were detected by gas chromatography-mass spectrometry using deuterium labelled sterols as internal standard[19,20,21].

Statistical analysis

Results are expressed as means ± standard deviation (SD). Statistical analyses were performed using Mann-Whitney U test. (Multiple) linear regression was used to correlate lipids and sterols with clinical variables and HbA_{1c}. Sterol/cholesterol concentrations, insulin dosage and HbA_{1c} were natural log transformed to obtain a normal distribution before statistical analysis. Level of significance for all statistical analyses was set at $p < 0.05$.

RESULTS

Serum cholesterol and triglyceride levels

Patient and control groups were comparable in age, gender, and BMI SDS (Table 1). Female patients with DM1 had significantly higher total cholesterol and LDL

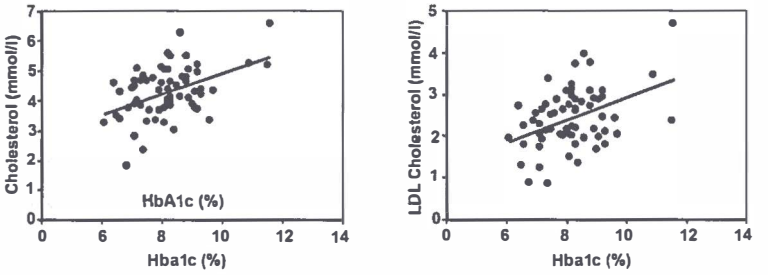

Figure 1. Non-fasting serum cholesterol and LDL cholesterol concentrations, compared with HbA_{1c} in patients with type 1 diabetes. Serum cholesterol concentration was correlated with HbA_{1c} with the following equation: [Serum cholesterol concentration] = 0.3408[HbA_{1c}] + 1.4491 ($r = 0.44$, $p < 0.001$). Serum LDL-cholesterol was correlated with HbA_{1c} with the following equation: [Serum LDL-cholesterol concentration] = 0.2668[HbA_{1c}] + 0.2462 ($r = 0.42$, $p < 0.001$).

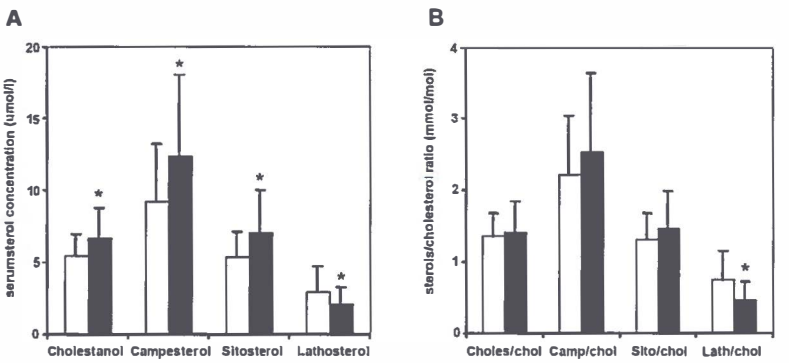

Figure 2. A: Cholestanol, campesterol and sitosterol (cholesterol absorption markers) and lathosterol (cholesterol synthesis markers) concentrations in patients with type 1 diabetes (closed bars) and controls (open bars). Values represent means ± SD. *: $p < 0.05$ compared to controls. **B:** Ratios of cholestanol, campesterol, sitosterol and lathosterol to cholesterol in patients with type 1 diabetes (closed bars) and controls (open bars). Values represent means ± SD. *: $p < 0.05$ compared to controls.

cholesterol concentrations than female controls (Table 1). Total cholesterol in female patients was also significantly higher compared with male patients, whereas body mass index SDS, HbA_{1c}, age, duration of diabetes and insulin dosage were comparable between female and male patients. LDL cholesterol concentrations in female controls were significantly lower compared with male controls, whereas body mass index SDS and age in female and male controls were comparable. In DM1 patients, linear regression analysis showed a significant correlation between HbA_{1c} and serum cholesterol, LDL cholesterol and the cholesterol/HDL cholesterol ratio (for cholesterol $r = 0.44$, $p < 0.001$, for LDL cholesterol $r = 0.42$, $p < 0.001$,

Figure 1, for cholesterol / HDL cholesterol r = 0.28, p < 0.05, data not shown). HDL cholesterol or triglycerides were not significantly correlated with HbA_{1c} (p > 0.05). Body mass index SD score for age and insulin dosage per kg body weight were not correlated with triglycerides, cholesterol, LDL cholesterol or HDL cholesterol concentrations. In DM1 patients, but not in controls, LDL cholesterol and cholesterol/HDL cholesterol ratio increased with advancing age (r = 0.29, p < 0.02; r = 0.33, p < 0.01, respectively).

Serum sterol concentrations

The serum lathosterol concentrations were significantly lower in DM1 patients compared with controls, while cholestanol and plantsterol concentrations (campesterol, sitosterol) were significantly higher in DM1 patients than in controls (Figure 2A). Figure 2B shows the ratios of cholestanol, campesterol, sitosterol and lathosterol to cholesterol in serum of DM1 patients and controls. The lathosterol / cholesterol ratio (reflecting cholesterol synthesis) was significantly lower in DM1 patients compared to controls. Ratios of cholestanol, campesterol, or sitosterol to cholesterol were not significantly different between DM1 patients and controls (p=0.24). Ratios of plantsterols and of lathosterol to cholesterol were not significantly correlated with gender, HbA_{1c}, body mass index SDS, or insulin dosage. Most importantly, the ratios of the cholesterol absorption markers (campesterol/cholesterol and sitosterol/cholesterol) were negatively correlated with the ratio of the synthesis marker (lathosterol/cholesterol) when calculated for all subjects (Figure 3).

In DM1 patients, markers of cholesterol absorption (Figure 4A) significantly decreased with age whereas the marker of cholesterol synthesis (Figure 4B) increased with age (p < 0.05). In controls, sitosterol/cholesterol ratios were

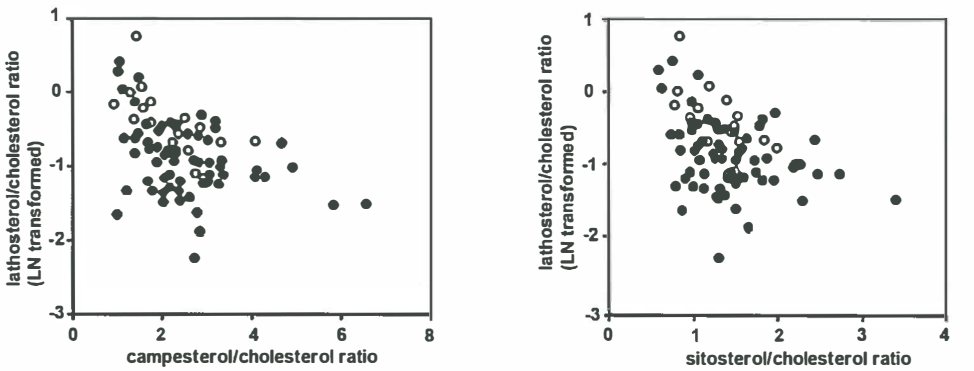

Figure 3. Relation of lathosterol (natural log transformed) and campesterol ratios in serum (r = -0.43, p < 0.0001) and of lathosterol (natural log transformed) and sitosterol ratios in serum (r = -0.37, p = 0.001). Open circles: controls, closed circles: DM1 patients.

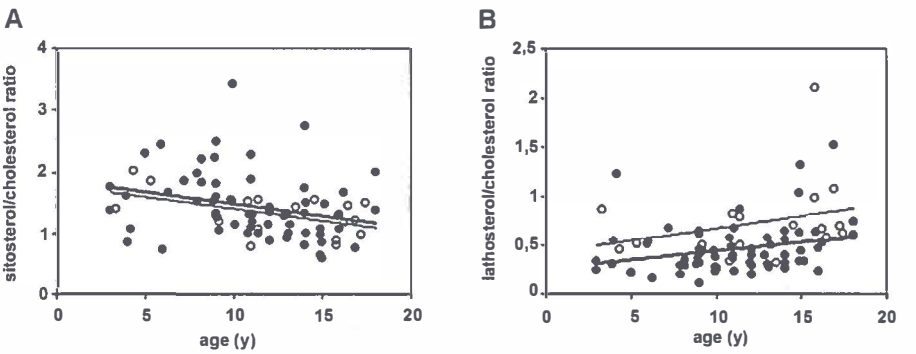

Figure 4. Sitosterol/cholesterol ratio and lathosterol/cholesterol ratio related to age in DM1 patients and controls. **A**: In DM1 patients sitosterol/cholesterol ratio was correlated with age with the following equation: [sitosterol/cholesterol ratio] = -0.0387[age] + 1.8671 (r = -0.27, p = 0.03). In controls: [sitosterol/cholesterol ratio] = -0.0385[age] + 1.7894 (r = -0.51, p = 0.035) **B:** In DM1 patients lathosterol/cholesterol ratio was correlated with age with the following equation: [lathosterol/cholesterol ratio] = 0.0178[age] + 0.2592 (r = 0.25, p = 0.04). In controls: [lathosterol/cholesterol ratio] = 0.0257[age] + 0.4147 (r = 0.29, p = 0.26). Closed circles, thick line: DM1 patients. Open circles, thin line: controls.

negatively correlated with age (r = -0.51, p < 0.05)(Figure 4B). Lathosterol/ cholesterol ratios in controls were not significantly correlated with age (p = 0.26)(Figure 4A). Multiple regression showed that both the presence of diabetes and age was associated with lathosterol/cholesterol ratios (r = 0.46, p < 0.01, beta −0.34, 0.26 respectively). In both patients and controls, age was not correlated with body mass index SDS. In DM1 patients, both HbA_{1c} and insulin dosage (U/kg/ day) were positively associated with age (r= 0.27, p < 0.05; r = 0.30, p < 0.05, respectively). DM1 patients with microalbuminuria (n=5), celiac disease (n=1), hypothyroidism (n=1) had comparable sterol/ cholesterol ratios compared with the other DM1 patients.

DISCUSSION

In the present study we aimed to identify if lower sterol markers for cholesterol synthesis and higher sterol markers for cholesterol absorption are present in children with DM1 compared with non-diabetic controls, comparable to the published data in adults with DM1[16]. In children and adolescents with DM1, serum lathosterol/cholesterol ratio, an established marker for cholesterol synthesis, was significantly decreased compared with age-matched controls. Sterol markers for cholesterol absorption, were significantly higher in DM1 patients compared with controls, although not when expressed relative to serum cholesterol concentrations. Lathosterol/cholesterol ratio showed a significant negative relationship with plantsterol/cholesterol ratios, suggesting that a lower rate of cholesterol synthesis

might be related to enhanced cholesterol absorption in children with DM1.

In children and adults with type 1 diabetes, elevated total cholesterol and LDL cholesterol in females compared with males has been described[22,23,24,25]. Our present data are in accordance with these observations: total cholesterol in female patients was higher compared with male patients ($p< 0.05$, Table 1), whereas BMI-SDS, HbA_{1c}, duration of diabetes and insulin dosage were comparable. Type 1 diabetes abolishes the gender difference in coronary heart disease mortality because it is associated with a greater increase of coronary disease in women than in men[26,27,28]. The pathophysiological basis of this is not understood: persistently elevated lipid levels in females with DM1 could be a contributing factor. Female patients with DM1 had significantly higher total cholesterol and LDL cholesterol than female age-matched controls, despite similar BMI-SD scores. Several studies in children with type 1 diabetes show higher cholesterol and LDL cholesterol concentrations compared with controls[24,29,30,31]. Improvement of metabolic control leads to lowering of cholesterol and LDL cholesterol levels[23,32,33]. It is possible that the relatively minor differences in plasma lipid levels in our study are related to relatively good metabolic control in our group.

The observed correlation between HbA_{1c} and serum cholesterol, LDL cholesterol and the cholesterol/HDL cholesterol ratio (Figure 1) is in agreement with previous studies in children and adolescents with type 1 diabetes, including of our own[17,22,23,29,34]. In DM1 patients, age was positively correlated with LDL cholesterol and cholesterol/HDL cholesterol ratio. Abraha et al. found no correlation between age and various lipid levels[34]. In the present study, age was also associated with increasing HbA_{1c} and increasing insulin dosage per kg bodyweight. Less favourable metabolic control and slight insulin resistance with advancing age may have contributed to the higher LDL cholesterol and cholesterol/HDL cholesterol ratio.

To our knowledge no data on cholesterol absorption and synthesis in children with DM1 have been published so far. In our young DM1 patients, quantification of serum sterol concentrations revealed significantly higher levels for markers of cholesterol absorption (cholestanol, campesterol and sitosterol) and lower levels for the established marker of cholesterol synthesis (lathosterol) compared with controls (Figure2A). When sterol concentrations were related to cholesterol, the lathosterol/cholesterol ratio was still significantly lower in DM1 compared with controls, whereas markers of cholesterol absorption were comparable ($P>0.05$, Figure 2B). Kojima, et al. described elevated plantsterol concentrations in adults with type 1 diabetes in poor control[14]. After improvement of glycemic control by intensive insulin therapy plantsterol concentrations were decreased to values comparable with controls[14]. Furthermore, Gylling et al. and Miettinen et al. showed that, in patients with DM1, markers of cholesterol absorption were increased and of cholesterol synthesis were reduced compared to patients with type 2 diabetes or controls[15,16]. Our data indicate that cholesterol synthesis is significantly correlated

with cholesterol absorption in children (Figure 3). These findings are comparable with the notion that enhanced intestinal cholesterol absorption leads to suppression of hepatic cholesterol synthesis in DM1. Reduced (hepatic) cholesterol synthesis, under most conditions, is associated with suppression of (hepatic) LDL-receptor expression and function[35]. Accordingly, cholesterol supplementation in adult DM1 patients raised serum LDL cholesterol more effectively than in age-matched controls, indicating that cholesterol absorption is probably higher in DM1[36].

In children with DM1, in contrast to Gylling et al. sterol/cholesterol ratios as markers for cholesterol absorption were comparable with controls. The reason for this is still unclear. Apart from the fact that our patients were much younger, the samples used were collected under non-fasting conditions, which may have influenced outcome. Furthermore, the controls in the Gylling study [16] were advised to take a low-saturated fat/low-cholesterol diet similar to their DM1 patients for at least 3 weeks before baseline. In our study, we had no information on the diet, however, excessive intake of dietary plantsterols that could have influenced serum plantsterol ratios was excluded[37]. On the other hand, it could be that at this age, cholesterol absorption is still unchanged likewise our previous study on chylomicron clearance rate in children with DM1[17]. Other factors may have influenced cholesterol concentration in children with DM1, for example a decreased LDL receptor expression[38,39].

In DM1 patients and in controls, age appeared to be significantly associated with increasing lathosterol and decreasing plantsterol levels (Figure 3). A study of Kempen et al described that absolute lathosterol concentrations were positively correlated with age and plantsterol concentrations negatively, only in boys[40]. These correlations appeared to be due to a correlation with weight. In our study insulin dosage (U/kg/day) was also positively correlated with age. This suggests that mild insulin resistance could have influenced the data. Accordingly, mild insulin resistance of puberty could play a role in the correlation found in controls[41,42]. Insulin resistance has been associated with increased cholesterol synthesis and decreased cholesterol absorption[43]. Therefore, the mild insulin resistance of puberty could contribute to the rising lathosterol- and decreasing plantsterol ratios with advancing age observed in patients and controls.

It is tempting to speculate which factors contribute to the changes in cholesterol metabolism observed in DM1 patients. We recently found an increased cholesterol absorption and a relatively decreased biliary cholesterol excretion associated with a decreased expression of intestinal and hepatic ATP binding cassette transporter g5 (Abcg5) and Abcg8[10] in STZ-diabetic rats. Reduced expression of these transporters might occur in humans with DM1 but, to the best of our knowledge, no data are available to substantiate this suggestion. Abcg5 and Abcg8 are target genes of the transcription factor Liver X receptor (LXR)[44]. Expression of LXR is stimulated by insulin and reduced LXR expression in the diabetic state might

alter expression of its target genes. Furthermore, metabolic consequences of DM1 (high concentrations of free fatty acids and ketone bodies) may interfere with LXR signaling, thereby causing the changes described above[45,46,47].

In conclusion, type 1 diabetes in children and adolescents is associated with decreased cholesterol synthesis and possibly increased cholesterol absorption. Cholesterol synthesis increases and cholesterol absorption decreases with age, possibly related to mild insulin resistance. Better understanding of cholesterol metabolism in type 1 diabetes may add to the development of therapeutic interventions at early age for decreasing the atherosclerotic risk later in life.

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4

Down-regulation of hepatic and intestinal Abcg5 and Abcg8 expression associated with altered sterol fluxes in rats with streptozotocin-induced diabetes

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ABSTRACT

Background/Aims: Type I diabetes is associated with altered hepatic bile formation and increased intestinal cholesterol absorption. The aim of this study was to evaluate whether altered expression of the ATP-Binding Cassette half-transporters Abcg5 and Abcg8, recently implicated in control of both hepatobiliary cholesterol secretion and intestinal cholesterol absorption, contributes to changed cholesterol metabolism in experimental diabetes.

Methods: mRNA and protein expression of *Abcg5* and *Abcg8* were determined in the liver and intestine of rats with streptozotozin-induced diabetes and related to relevant metabolic parameters in plasma, liver and bile.

Results: Hepatic mRNA expression of both *Abcg5* (-76%) and *Abcg8* (-71%) was reduced in diabetic rats when compared to control rats. In spite of increased HDL cholesterol, considered a major source of biliary cholesterol, secretion of the sterol into bile relative to that of bile salts was reduced by 65% in diabetic animals. Intestinal mRNA expression of *Abcg5* (-47%) and *Abcg8* (-43%) as well as Abcg5 protein contents were also reduced in insulin-deficient animals. This was accompanied by a three- to four-fold increase in plasma β-sitosterol and campesterol concentrations and by a doubling of the calculated apparent cholesterol absorption. These effects partially normalized upon insulin supplementation.

Conclusions: Our data indicate that effects of insulin-deficiency on bile composition and cholesterol absorption in rats are, at least partly, attributable to changes in hepatic and intestinal *Abcg5* and *Abcg8* expression.

INTRODUCTION

Type 1 diabetes mellitus is associated with specific changes in cholesterol metabolism in humans[1] and in experimental animals[2, 3], including increased concentrations of plasma cholesterol, enhanced conversion of cholesterol into bile salts and an enhanced intestinal cholesterol absorption. The hepatobiliary pathway is of crucial importance for the maintenance of cholesterol homeostasis[4]. Bile salts that are secreted by the liver into the intestinal lumen are required for intestinal absorption of dietary cholesterol. The majority of bile salts is subsequently reabsorbed from the intestine and returns to the liver for re-secretion into the bile. The relatively small fraction of bile salts that escapes intestinal absorption is compensated for by de novo synthesis from cholesterol in the liver. Secondly, bile contains considerable amounts of free cholesterol. Since only a part of biliary cholesterol is reabsorbed from the intestine[5], the biliary pathway contributes to a major extent to cholesterol turnover. It is well-established that secretion of cholesterol into bile is coupled to that of phospholipids in a process that is, in part, controlled by bile salt secretion[6]. Recent studies, however, indicate that specific ABC transporters, i.e., Abcg5 and Abcg8, are involved in biliary cholesterol secretion[7, 8]. The genes encoding these transporters are highly expressed in the liver[9]. Mutations in the human genes encoding ABCG5 and ABCG8 have been shown to cause sitosterolaemia[10, 11, 12] with a reduced biliary secretion as well as a strongly enhanced intestinal absorption of plant sterols (sitosterol, campesterol). Indeed, ABCG5 and ABCG8 are also highly expressed in the intestine[9] and supposedly involved in efflux of plant sterols taken up by enterocytes back into the intestinal lumen, thereby preventing absorption. Based on the fact that the efficiency of dietary cholesterol absorption is high in sitosterolaemia patients, a role of ABCG5 and ABCG8 in the control of cholesterol absorption efficiency has been proposed[10, 11, 12]. Accordingly, cholesterol absorption was reduced in mice over-expressing both transporters[7] and in mice in which expression of the transporters was induced by pharmacological means.

Type 1 diabetes is associated with altered expression of several ABC transporters in the liver. In a recent study[14], we showed that streptozotocin (STZ)-induced diabetes in rats differentially affects the expression of hepatic ABC transporters which, at least in part, underlie reported effects on bile composition. Specifically, we found a very strong up-regulation of *Abcb4* (multidrug resistance P-glycoprotein subtype 2 or Mdr2) mRNA and Abcb4 protein, in accordance with a strong induction of biliary phospholipid secretion. In spite of the characteristic increase in biliary bile salt output rates, we found no effects on Abcb11 (bile salt export pump or Bsep) protein content in livers of STZ-diabetic rats.

To investigate whether changes in *Abcg5/Abcg8* expression contribute to the established effects of insulin deficiency on cholesterol metabolism, we have determined their mRNA abundances and protein contents in the liver and intestine

of rats with STZ-diabetes and related these to the actual sterol fluxes. Our data indicate that the suppressive effects of insulin-deficiency on biliary cholesterol secretion and its stimulatory effects on cholesterol absorption in rats are, at least in part, attributable to changes in hepatic and intestinal *Abcg5* and *Abcg8* expression.

MATERIALS AND METHODS

Animals

Male Wistar rats (260-300g) were purchased from Harlan (Zeist, The Netherlands) and housed in a temperature-controlled environment with alternating 12-h light and dark periods. The rats received standard laboratory chow (RMH-B; Hope Farms BV, Woerden, The Netherlands) and had free access to food. Experimental procedures were approved by the local Ethics Committee for Animal Experimentation.

Experimental procedures

Diabetes was induced by a single intraperitoneal injection (60 mg/kg body weight) of STZ (Pharmacia & Upjohn, Kalamazoo, Mich., USA). Control animals received an injection of the solvent (sodium citrate, 3% w/v). Induction of diabetes was perceived by development of hyperphagia, polydipsia and polyuria and confirmed by determination of the degree of hyperglycemia. Three weeks after STZ injection, one half of the diabetic group was treated with subcutaneously administered insulin (long acting insulin, Humuline NPH, Eli Lilly, Nieuwegein, The Netherlands, 1 IU in the morning and 2 IU in the evening). Experiments were carried out at 4 weeks after STZ injection. Food intake was monitored by weighing of food containers and faeces was collected quantitatively during the last 3 days prior to death of the animals. At that time, six control, six diabetic and six diabetic insulin-treated rats, were anaesthesized with pentobarbital (60 mg/kg body weight) and bile was collected for 30 min upon cannulation of the bile duct. Blood samples were collected by heart puncture, transferred to EDTA-containing tubes and centrifuged immediately (10 000 g). Plasma was stored at –20 °C until analyses and the livers were rapidly excised and weighed. Parts of the liver were snap-frozen in liquid nitrogen for RNA isolation, isolation of plasma membrane fractions or determination of lipid levels. The small intestines were flushed with a buffered salt solution; representative parts of the intestine were snap-frozen in liquid nitrogen for RNA isolation or isolation of brush border membranes.

Analytical procedures

Plasma concentrations of total cholesterol, triglycerides and free fatty acids were measured with commercially available kits (Roche, Mannheim, Germany, or Wako, Neuss, Germany)[13, 15]. Pooled plasma samples of the three groups of

rats were used for lipoprotein separation by fast protein liquid chromatography (FPLC)[15]. Plasma plant sterol and cholesterol concentrations were determined by gaschromatography[16].

Biliary bile salt concentrations were measured enzymatically[13, 15]. Hepatic and biliary phospholipids and cholesterol lipid contents were measured after extraction[17, 18, 19]. Faecal and chow contents of neutral sterols were measured as described[15].

Western blotting

Plasma lipoprotein fractions separated by FPLC were taken for semi-quantitative assessment of apoA-I contents by Western blotting[15].

Hepatic plasma membrane fractions were prepared and characterized as described[20]. Proteins from liver homogenates (75 ug protein/lane) or liver plasma membranes (10 ug protein/lane) were separated on 4-15% Ready Gels (Bio-Rad Laboratories, Hercules, Calif., USA) and blotted onto nitrocellulose membranes by tank blotting. Membranes were blocked overnight in a 5% skimmed milk powder solution in Tris-buffered saline containing 0.1% Tween 20 (TTBS) and subsequently incubated with the primary antibody (rabbit polyclonal anti-SR-BI, Novus Biologicals, Littleton, Colo., USA, NB400-101) diluted 1:20 000 in TTBS for 1h at room temperature. After washing anti-rabbit IgG linked to horseradish peroxidase, diluted 1:1000 in TTBS, was added for 1 h. Detection was carried out using ECL, according to the manufacturer's instructions (Amersham, Roosendaal, the Netherlands).

Proteins from intestinal brush border membranes, isolated as described previously[20], were separated on 8% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked in TTBS and subsequently incubated for 1 h at room temperature with primary antibodies, raised in rabbits against amino acids 256-392 of murine Abcg5[21], diluted 1:1000 in blocking buffer. Membranes were washed thrice in TTBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Bio-Rad) diluted 1:2000 in blocking buffer. Membranes were washed four times in TTBS and bands were visualized using Lumi-light^{plus} Western blotting substrate in a Lumi-Imager F1 workstation (Roche).

RNA isolation and RT-PCR procedures

RNA isolation and cDNA synthesis were carried out as described[22]. Real-time quantitative PCR was done as described[23] and modified in our laboratory[13]. Primer and probe sequences (Invitrogen, Carlsbad, USA) and detection probes (Eurogentec, Seraing, Belgium) for the genes of interest, labelled with the 5' linked fluorescent reporter dye 6-carboxy-fluorescein (FAM) and the 3'linked fluorescent quencing dye 6-carboxy-tetramethyl-rhodamine (TAMRA), have been published[13]. Measurements were done using an ABI Prism 7700 Sequence Detector with 1.6.3

software (Perkin-Elmer Corp., Foster City, Calif., USA).

Statistics

Results are presented as mean values ± SD. Statistic analyses were carried out using one-way ANOVA with Bonferroni correction or, when two groups were compared, by the Mann-Whitney U test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Animal characteristics

STZ rats had lower body weights than control rats at the end of the experiment (-29%, $p < 0.05$), which did not normalize upon treatment with insulin (-25%). The ratio liver-to-body weight was increased by 37% ($p < 0.05$) upon STZ treatment and this effect did not disappear after insulin treatment. Blood glucose concentrations were higher in diabetic rats than in controls, i.e., 23.2 ± 3.5 compared with 5.8 ± 0.3 mmol/L ($p < 0.05$), whereas those in insulin-treated diabetic rats were intermediate (11.9 ± 5.4 mmol/L).

Plasma cholesterol, triglyceride, and free fatty acid concentrations were all increased in diabetic rats and showed a tendency towards normalization in insulin-treated diabetic rats (Table 1). FPLC separation of plasma lipoproteins showed that the increase in plasma cholesterol in diabetic animals was due to increases in VLDL-, LDL- as well as HDL-sized fractions (Figure 1A), whereas the increase in triglycerides was exclusively in VLDL-sized fractions (Figure 1B). Western blot analysis of FPLC fractions revealed a higher apolipoprotein (apo) A-I content in HDL fractions of diabetic rats than in those of control rats (Figure 1C). Hepatic mRNA abundance of *Apoa-I* was more than two-fold induced in diabetic rats and normalized upon insulin treatment (data not shown).

No differences in hepatic free cholesterol contents were noted between control, diabetic and insulin-treated diabetic animals, i.e, 25.5 ± 1.4, 24.0 ± 3.6 and 25.5 ± 4.5 nmol/mg protein, respectively. As expected, expression of sterol regulatory

Table 1: Plasma concentrations of cholesterol, triglycerides and free fatty acids in control, diabetic and insulin-treated diabetic rats

	Control	Diabetes	Diabetes + Insulin
Cholesterol (mmol/L)	1.62 ± 0.13	2.59 ± 0.48*	2.11 ± 0.56*
Triglycerides (mmol/L)	1.29 ± 0.60	5.99 ± 2.29*	3.61 ± 1.47*
Free fatty acids (µmol/L)	231 ± 32	572 ± 184*	406 ± 107*

Mean values ± SD are shown for 6 animals per group. Asterisks indicate significant difference from control values.

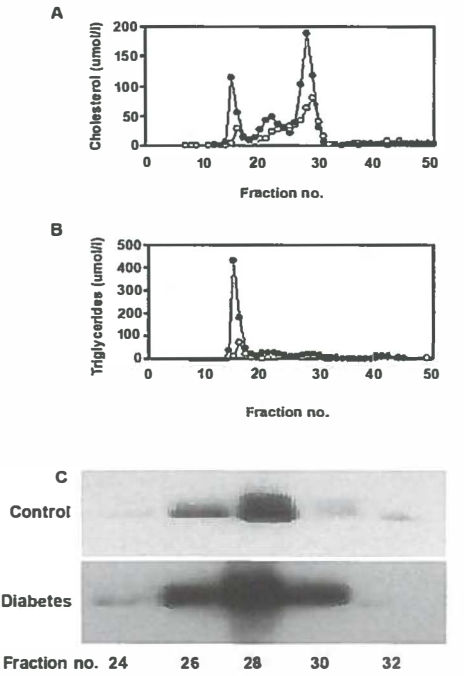

Figure 1 Effects of streptozotocin-induced diabetes on distribution of (**A**) cholesterol, (**B**) triglycerides and (**C**) apolipoprotein A-I in plasma lipoprotein fractions. *Open symbols*, control rats; *closed symbols*, diabetic rats. Diabetic rats treated with insulin showed concentrations of cholesterol and triglycerides that were intermediate between those of controls and untreated diabetic rats across all lipoprotein fractions: these data are not shown for reasons of clarity. *Top panel C*, control rats; *bottom panel C*, diabetic rats

element binding protein 1c (*Srebp1c*) was strongly reduced in diabetic animals whereas that of *Srebp2*, the transcription factor that is primarily responsible for control of genes involved in maintenance of hepatocytic cholesterol homeostasis, remained unaffected (Figure 2). Expression of genes encoding proteins involved in cholesterol synthesis (HMG CoA reductase, *Hmgr*) and lipoprotein uptake (LDL receptor, *Ldlr*) were down-regulated in diabetic animals, whereas that of acyl-CoA cholesterol acyl transferase 2 (*Acat2*), involved in cholesterol esterification, was not affected (Figure 2).

Reduced hepatic expression of Abcg5 and Abcg8 is associated with impaired hepatobiliary cholesterol transport

mRNA abundances of *Abcg5* and *Abcg8* were strongly reduced in the livers of diabetic rats in comparison to those in control animals. This consequence of long-term insulin-deficiency did not normalize upon treatment of diabetic rats with insulin. Both *Abcg5* and *Abcg8* gene expression are controlled by the liver X-

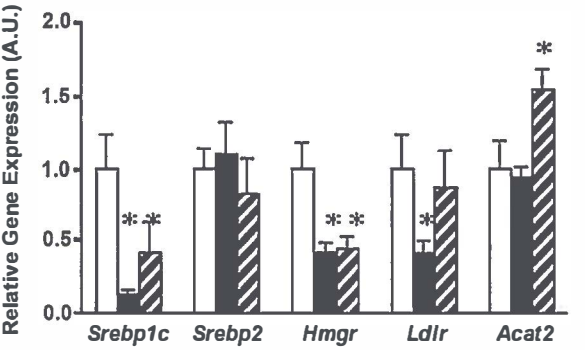

Figure 2 Changes in relative hepatic gene expression of *Srebp1c*, *Srebp2*, *Hmgr*, *Ldlr*, and *Acat* 2 upon induction of streptozotocin-diabetes, determined by realtime PCR *Open bars*, control rats; *closed bars*, diabetic rats; *striped bars*; diabetic rats treated with insulin. Mean values ± SD of 4–6 rats per group, asterisks indicate significant difference from control values

receptor: no differences in expression of the gene encoding the most abundant isoform of this transcription factor in the liver, i.e., *Lxrα*, were noted between the groups (Figure 3).

To test the functional consequences of reduced hepatic *Abcg5* and *Abcg8* expression, we measured biliary cholesterol output in rats of the three experimental groups. Cholesterol output in the three groups of animals was expressed relative to that of bile salts (Figure 4A) and to that of phospholipids (Figure 4B). It is evident that diabetic rats secreted much less cholesterol relative to bile salts and to phospholipids than control rats did: insulin treatment failed to completely restore hepatobiliary cholesterol hyposecretion.

HDL is considered to be an important source of biliary cholesterol and concentrations of plasma HDL cholesterol were clearly elevated in diabetic

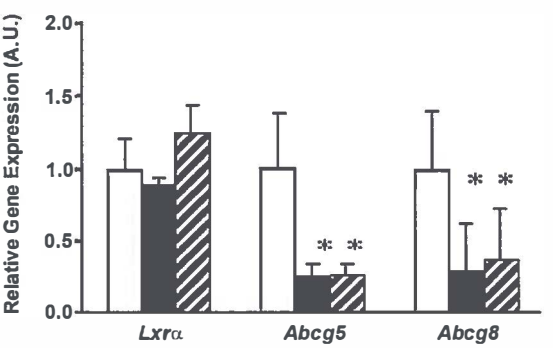

Figure 3 Changes in relative hepatic gene expression of *Lxrα, Abcg5* and *Abcg8* upon induction of streptozotocin-diabetes in rats, determined by realtime PCR. *Open bars*, control rats; *closed bars*, diabetic rats; *striped bars*; diabetic rats treated with insulin. Mean values ± SD of 6 rats per group, asterisks indicate significant difference from control values

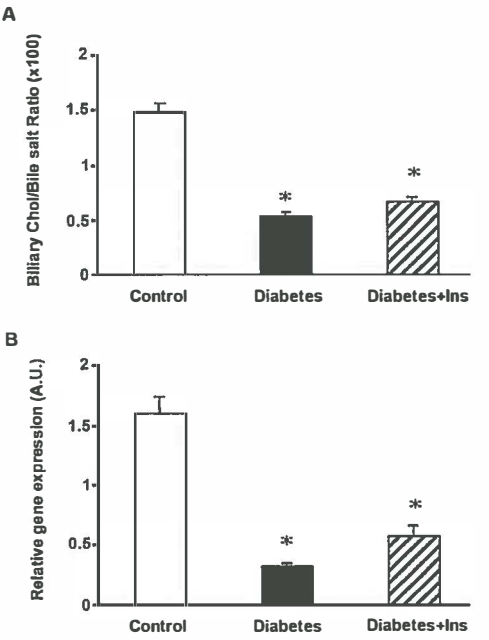

Figure 4 Changes in biliary cholesterol content upon induction of streptozotocin-diabetes in rats. Biliary cholesterol concentration was expressed relative to that of (**A**) bile salts or (**B**) phospholipids. *Open bars*, control rats; *closed bars*, diabetic rats; *striped bars*; diabetic rats treated with insulin. Mean values ± SD of 6 rats per group, asterisks indicate significant difference from control values

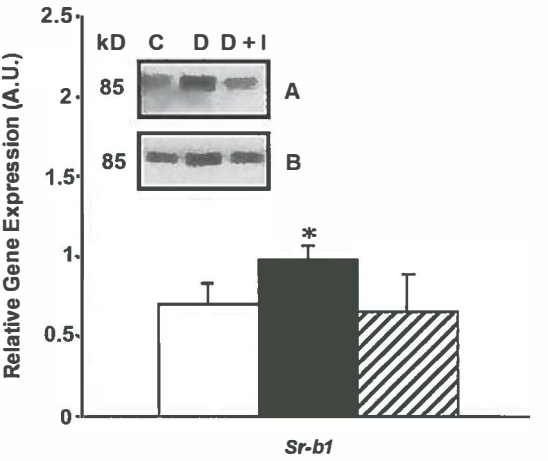

Figure 5 Effects of streptozotocin-diabetes on hepatic mRNA and protein expression of SR-BI. *Open bars*, control rats; *closed bars*, diabetic rats; *striped bars*; diabetic rats treated with insulin. Mean values ± SD of 6 rats per group, asterisks indicate significant difference from control values. Inserts show corresponding levels of SR-BI protein in (**A**) liver homogenates and (**B**) plasma membrane fractions. Data shown are representative examples of at least 4 preparations per group. *C*, control; *D*, diabetic rats; *D + I*, diabetic rats + insulin

rats (Figure 1). To evaluate whether a reduced hepatic uptake capacity of HDL cholesterol (ester) might contribute to biliary cholesterol hyposecretion, we analysed hepatic mRNA and protein expression of the major HDL-receptor, i.e., scavenger receptor class B type 1 (SR-BI) in livers of the three groups of rats. mRNA abundance of *Sr-bI* was slightly higher in diabetic rats than in controls and normalized upon insulin treatment (Figure 5). SR-BI protein content was clearly increased in liver homogenates of diabetic rats compared to those of controls and insulin-treated diabetic rats (Figure 5A) while the amounts of the protein in hepatic plasma membrane fractions were rather similar among the three groups (Figure 5B).

Reduced intestinal expression of Abcg5 and Abcg8 is associated with enhanced cholesterol absorption

mRNA expression levels of *Abcg5* and *Abcg8* were reduced by 47% and 43% in the jejunum of diabetic rats when compared to controls and normalized upon insulin treatment (Figure 6). The expression of *Abca1*, also implicated in the control of intestinal cholesterol absorption, showed a similar pattern as those of *Abcg5* and *Abcg8*. Intestinal expression of *Lxrα* tended to be reduced in diabetic rats and normalized upon insulin treatment.

Reduced mRNA abundance was associated with a clearly reduced protein content of Abcg5 in brush border membrane preparations isolated from the small

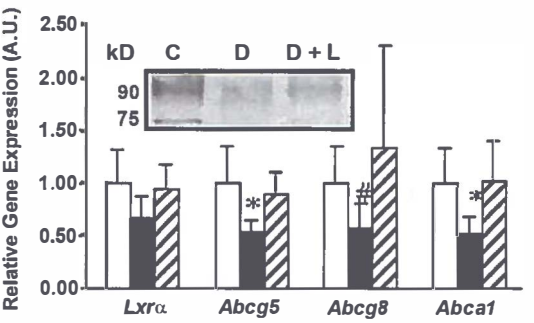

Figure 6 Changes in relative intestinal gene expression of *Lxrα*, *Abcg5*, *Abcg8* and *Abca1*, determined by realtime PCR, and intestinal Abcg5 protein content upon induction of streptozotocin-diabetes in rats. *Open bars*, control rats; *closed bars*, diabetic rats; *striped bars*; diabetic rats treated with insulin. Mean values ± SD of 6 rats per group, asterisks indicate significant difference from control values, #indicates p=0.059. Insert shows Abcg5 protein levels in brush border membranes from jejunal sections of rats of the three groups. Bands at 90 and 75 kD reacted to the antibody raised against mouse Abcg5. Competition with the peptide used to raise the antibody strongly decreased these signals. Incubation of the protein fractions with N-glycosidase F to remove all N-linked sugar chains decreased the apparent molecular weight of both bands to a single band of ~64 kD. *C*, control; *D*, diabetic rats; *D + I*, diabetic rats + insulin

intestines of diabetic rats (Figure 6).

To assess the functional consequences of reduced *Abcg5* and *Abcg8* expression in the intestines of diabetic rats, plasma concentrations of the plant sterols campesterol and β-sitosterol were determined. These sterols are considered natural substrates of Abcg5/Abcg8: their concentrations are strongly increased in sitosterolaemia patients and have been advocated as an indirect measure of cholesterol absorption efficicency. The concentration of both sterols was increased in diabetic rats when compared to control values and decreased upon insulin treatment of diabetic animals (Figure 7A). Plasma plant sterol concentrations remained increased in diabetic rats when normalized to plasma cholesterol concentrations (Figure 7B). The difference between calculated daily

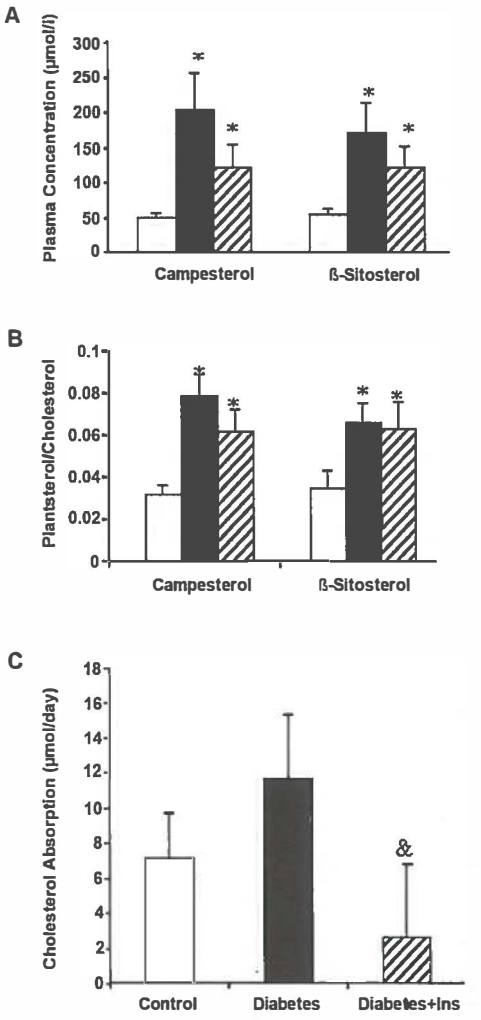

Figure 7 Changes in indices of intestinal cholesterol absorption efficiency upon induction of streptozotocin-diabetes in rats. (**A**) Plasma concentrations of campesterol and β-sitosterol were measured in control rats, diabetic rats and diabetic rats treated with insulin and (**B**) normalized to that of cholesterol, to exclude that the observed changes in plasma plant sterols were due to a-selective alterations in plasma sterol levels. (**C**) Apparent cholesterol absorption was calculated for the three groups by subtraction of the measured daily faecal neutral sterol loss from the estimated daily input. *Open bars*, control rats; *closed bars*, diabetic rats; *striped bars*; diabetic rats treated with insulin. Mean values±SD of 6 rats per group, asterisks indicate a significant difference from control values, & indicates significant difference from diabetes group

biliary excretion rates of cholesterol and an estimation of dietary cholesterol intake (input) and daily faecal neutral sterol loss (output) were used to calculate the "apparent cholesterol absorption" in the three groups of animals (Figure 7C). It was found that this calculated value was increased in the diabetic animals in comparison to controls, while it decreased again after treatment of diabetic animals with insulin.

DISCUSSION

The results show that STZ-induced Type I diabetes in rats is associated with reduced hepatic and intestinal expression of the ABC half-transporters Abcg5 and Abcg8. Reduced hepatic expression of these "sitosterolaemia genes" coincided with a reduction of hepatobiliary cholesterol secretion, whereas their reduced intestinal expression was associated with an increased absorption of cholesterol as deduced from an indirect measure of the process (plasma plant sterol concentrations) and from calculation of the apparent absorption efficiency. Similar changes in cholesterol transport have been reported in sitosterolaemia patients[24], and in Abcg5/Abcg8-deficient mice[8]. Over-expression of the human genes in transgenic mice[7] and pharmacological induction of expression of the endogenous genes in wild type mice[13] have been reported to have opposite effects, i.e., to stimulate biliary cholesterol excretion and to reduce intestinal cholesterol absorption. Consequently, it is highly likely that effects of insulin-deficiency on cholesterol metabolism are, at least in part, caused by the changes in *Abcg5* and *Abcg8* expression.

Recently, two groups have independently identified mutations in either *ABCG5* or *ABCG8* as the cause of the rare, recessively inherited metabolic disease sitosterolaemia[10, 11, 12]. Patients with this disease develop xanthomas and premature atherosclerosis[24, 25]. Affected individuals show high concentrations of plant sterols in plasma due to the fact that, in contrast to healthy subjects, they efficiently absorb these sterols from the intestine and are unable to excrete them into the bile[24, 26]. Sitosterolaemia patients have also been reported to efficiently absorb dietary cholesterol and to show impaired biliary cholesterol excretion[26, 27]. *ABCG5/Abcg5* and *ABCG8/Abcg8* are predominantly expressed in hepatocytes and in small intestinal enterocytes in humans and mice. The two genes are arranged in a head-to-head configuration in the human[28] and mouse[9] genome. Expression of both genes is co-ordinately regulated and highly induced in mice kept on a high-cholesterol diet[29]. LXRα, a nuclear receptor activated by oxysterols that plays a crucial role in regulating genes involved in cholesterol trafficking[30], is required for induction of murine *Abcg5* and *Abcg8* expression upon cholesterol feeding[29]. Treatment of mice with synthetic LXR agonists strongly induces the expression of both genes in liver and intestine[13, 29]. Recent studies[31], in which epitope-tagged

mouse Abcg5 and Abcg8 were expressed in cultured cells, have shown that heterodimerization of these half-transporters is required for their transport from the endoplasmic reticulum to the apical plasma membrane. Thus, available data indicate that the Abcg5/Abcg8 heterodimer is present at the canalicular membrane of hepatocytes where it is involved in secretion of cholesterol and plant sterols into the bile. In the intestine, the heterodimer seems to promote the efflux of (dietary) sterols, taken up by the enterocytes by as yet unidentified mechanisms, back into the lumen and thereby reduce the efficiency of their absorption. Overall, the physiological action of the transporter complex limits accumulation of sterols in the body.

Under normal conditions, biliary secretion of cholesterol is tightly coupled to that of phospholipids in a process controlled by bile salt secretion[6]. Accordingly, one would expect biliary cholesterol secretion to be enhanced in diabetic rats. This was evidently not the case: in spite of a strong increase in biliary bile salt and phospholipid secretion[14], diabetic rats displayed a relative hyposecretion of biliary cholesterol. Theoretically, hyposecretion could be explained by a lack of bile-destined cholesterol in the liver. It has been proposed that HDL cholesterol is a primary source of biliary cholesterol after its selective uptake by SR-BI[32]. One study[33] reported biliary cholesterol hyposecretion in SR-BI-deficient mice while another[34] reported hypersecretion in mice with hepatic SR-BI over-expression. We found that plasma HDL cholesterol and apo A-I levels were increased in diabetic rats, as is the case in SR-BI-deficient mice[33]. Yet, hepatic *Sr-bI* mRNA expression was up-regulated and corresponding protein contents were clearly increased in liver homogenates. SR-BI protein remained largely unaffected in plasma membrane fractions isolated from livers of diabetic rats, suggestive for altered sorting. Taken together, our data do not support impaired SR-BI-mediated HDL uptake as a cause of cholesterol hyposecretion in diabetic rats. In fact, our recent observation that cholesterol secretion is unaffected in Abca1 null mice lacking HDL[35] strongly argues against a regulatory role of cholesterol delivery. A concise overview of various models of cholesterol hypo- and hypersecretion indicated that, at least in mice, biliary cholesterol secretion strongly correlates with hepatic *Abcg5/Abcg8* expression[21]. Accordingly, we propose that impaired hepatic expression of both half-transporters underlies impaired cholesterol secretion in diabetic rats. Insulin treatment of diabetic rats did not normalize hepatic Abcg5/Abcg8 expression and, consequently, failed to normalize cholesterol secretion into bile.

The intestine is another site where Abcg5/Abcg8 exert control on cholesterol metabolism, i.e., by regulating the efficiency of cholesterol absorption. It is known that cholesterol absorption is increased in chronically diabetic rats[2, 3], but underlying mechanisms have remained elusive so far. Our study confirms the increased cholesterol absorption in STZ-treated rats by two independent methods. We found that plasma concentrations of β-sitosterol and campesterol were three-

to four-fold higher in diabetic rats than in controls and tended to normalize upon treatment of diabetic rats with insulin. Furthermore, we have calculated the apparent absorption by subtracting daily faecal neutral sterol output from the estimated daily input of dietary and biliary cholesterol. Although this calculation is based on a number of assumptions, the results strongly suggest that the apparent absorption is increased in diabetic animals. Intestinal hypertrophy[36] and the enlarged bile salt pool[3] have been proposed as rather unspecific causes of enhanced cholesterol absorption in experimental diabetes. Furthermore, cholesterol esterification by the enzyme acyl-CoA cholesterol acyltransferase, producing cholesteryl esters that can be incorporated into chylomicrons, has been shown to be enhanced in diabetic animals[37]. Our data show that reduced expression of *Abcg5* and *Abcg8* in the intestine of diabetic rats could contribute to the enhanced cholesterol absorption associated with this condition.

The obvious question concerns the cause of *Abcg5/Abcg8* down-regulation in liver and intestine of insulin-deficient rats. As mentioned previously, both genes are under control of LXRα, a sterol-sensing nuclear receptor that, upon dimerization with activated RXR, induces transcription of a large number of genes involved in cholesterol trafficking[30]. We found no changes in mRNA expression of *Lxrα* itself or of *Rxrα*, either in the liver or intestine. *Lxrα* expression was reported to be induced in cultured hepatocytes exposed to insulin and in livers of rats and mice upon acute administration of insulin[38]. This suggests that, in our chronic model of insulin deficiency, alternative modes of regulation maintain hepatic *Lxrα* expression. This obviously does not exclude the possibility of reduced LXRα protein concentrations or reduced amounts of its most potent ligands, i.e., (24S), 25-epoxycholesterol, (24S)-hydroxycholesterol, or (22R)-hydroxycholesterol. Alternatively, it could be that metabolic consequences of diabetes interfere with LXRα signalling. In a recent paper[39], evidence was provided to suggest that reduced expression of another important ABC transporter, i.e., Abca1, in liver and macrophages of diabetic mice is due to the characteristically high concentrations of free fatty acids and ketone bodies (particularly acetoacetate). In this study, we have confirmed down-regulation of *Abca1* expression in the liver of diabetic rats and show that expression of this gene is also strongly reduced in their small intestine. Expression of *Abca1* is also under control of LXRα[30] and unsaturated fatty acids have been shown to antagonize activation of this nuclear receptor by oxysterols so that transcription of target genes is inhibited[40, 41]. The mechanism by which acetoacetate interferes with ABC transporter expression is fully elusive at the moment but, in view of the similar mode of regulation of *Abca1* and *Abcg5/Abcg8* genes[30], it could well be involved in the down-regulation of *Abcg5/Abcg8* observed in this study. Thus, impaired *Abcg5/Abcg8* expression in liver and intestine in diabetes may be related to the accelerated lipolysis and/or increased ketogenesis that is associated with this condition. A direct role of insulin-deficiency per se is not likely, since we found

no differences in *Abcg5/Abcg8* expression between rat hepatocytes cultured for up to 24 h in the absence or presence of insulin.

In conclusion, we have provided evidence that suppression of *Abcg5* and *Abcg8* expression in the liver and intestine contributes to altered hepatobiliary and intestinal sterol fluxes that collectively promote accumulation of these sterols in the body in diabetic rats. When similar events occur in human diabetics, as suggested by increased plasma levels of plant sterols in subjects with poorly controlled Type I diabetes[42], this could contribute to an enhanced risk for development of atherosclerosis[43].

Acknowledgements

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Differential effects of streptozotocin-induced diabetes on expression of hepatic ABC-transporters in rats.

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ABSTRACT

Background/Aims: Diabetes mellitus is associated with changes in bile formation. The aim of our study was to investigate the molecular basis for these changes in rats with experimentally-induced diabetes.

Methods: Expression of bile canalicular transporters was studied by reverse-transcription polymerase chain reaction, immunoblotting and immunohistochemistry in control, streptozotocin-diabetic and insulin-treated diabetic rats. Bile formation was studied under basal conditions and during stepwise increasing i.v. infusion of taurocholate to determine bile salt secretory rate maximum (SRm).

Results: In diabetic rats, hepatic gene and protein expression of the multidrug resistance P-glycoprotein type 2 (Mdr2-Pgp) were increased by 105% and 530% respectively, associated with increased biliary phospholipid output (+520%) and phospholipid/bile salt ratio (+77%). Protein levels of the canalicular bile salt export pump (Bsep) were unchanged in diabetic rats, but basal biliary bile salt output and the SRm of taurocholate were increased by 260% and 130%, respectively, compared with controls. Alterations in transporter expression and bile formation were partly reversed by insulin administration. The bile salt SRm was strongly correlated with biliary phospholipid concentration ($p < 0.001$, $R = 0.82$).

Conclusions: Induction of *Mdr2* expression and biliary phospholipid secretion, rather than *Bsep* expression, appears to be responsible for the enhanced capacity of biliary bile salt secretion in experimentally-induced diabetes.

INTRODUCTION

Diabetes mellitus is associated with specific changes in bile formation in humans[1,2] and in experimental animals[3,4]. Increased biliary bile salt and phospholipid output rates as well as increased bile salt pool size have been described in rats with streptozotocin (STZ)- or alloxan-induced diabetes[3-5]. In diabetic rats, the bile salt pool contains an increased percentage of cholate, indicative for altered bile salt synthesis[6,7]. This is in accordance with the well-established suppressive effect of insulin on activity of enzymes involved in bile salt synthesis[8]. The secretory rate maximum (SRm) of bile salts, as determined during intravenous bile salt infusions at supraphysiological rates, seemed to be higher in alloxan-induced diabetic rats than in controls[5]. In contrast, the biliary secretion of other biliary constituents, including glutathione, was decreased in diabetic rats[9]. The mechanisms responsible for these changes in bile formation and composition in experimentally-induced diabetes are still largely unknown.

Recently, several adenosine triphosphate (ATP)-binding cassette (ABC) transporter proteins involved in hepatobiliary transport of bile constituents have been identified (see Koopen et al.[10] for review). Biliary phospholipid secretion is controlled by the multidrug resistance P-glycoprotein type 2 (Mdr2-Pgp) or, according to the most recent nomenclature, Abcb4 in rodents, and by its homologue MDR3 (=ABCB4) in humans[11,12]. The canalicular bile salt export pump (Bsep), also called sister of P-glycoprotein (Spgp) or Abcb11, has been identified as the major canalicular bile salt transporting protein[13]. The multidrug resistance-associated protein 2 (Mrp2), previously known as canalicular multispecific organic anion transporter and in the most recent nomenclature Abcc2, is, amongst other functions, involved in biliary secretion of glutathione[14].

The aim of the present study was to elucidate the effects of STZ-induced diabetes in rats on the expression of specific hepatic ABC-transporter proteins that are involved in the transport of biliary constituents (i.e., Mdr2-Pgp, Bsep and Mrp2). The changes in transporter protein expression were related to changes in bile formation in STZ-induced diabetic rats. Our results show that STZ-induced diabetes in rats is associated with a pronounced induction of Mdr2-Pgp protein expression, a strong reduction of Mrp2 expression, whereas Bsep expression remains unaffected. These effects were partially reversed by insulin administration. Our data are compatible with the concept that bile salt SRm in rats is not controlled by Bsep protein expression, but rather by biliary phospholipid concentration and thus by Mdr2-Pgp activity.

MATERIALS AND METHODS

Animals

Male Wistar rats (260-320g) were purchased from Harlan (Zeist, the Netherlands) and housed in a temperature-controlled environment with alternating 12-hour light and dark. The rats received standard laboratory chow (RMH-B; Hope Farms BV, Woerden, the Netherlands) and had free access to food and water. All experiments were performed according to the institutional guidelines for the care and use of laboratory animals in research.

Experimental design and animal treatments

Three independent experiments were performed. In all experiments, diabetes was induced by a single intraperitoneal injection (6 mg/100 g body weight [BW]) streptozotocin (Pharmacia & Upjohn, Kalamazoo, MI). Control animals received an intraperitoneal injection of the solvent (3% sodium citrate). Induction of diabetes was perceived by development of hyperphagia, polydipsia, and polyuria and confirmed by determination of the degree of hyperglycemia. Three weeks after STZ injection, groups of diabetic rats were treated with subcutaneously administered insulin (long acting insulin [Humuline NPH, Eli Lilly, Nieuwegein, the Netherlands]; 1 IU in the morning and 2 IU in the evening; STZ-Ins rats). Experiments were performed at 4 weeks after STZ injection. No rats receiving STZ died during the 4 weeks of the experiment.

Bile salt pool size and synthesis rate

At 3 weeks after STZ injection, catheters were placed in the common bile duct and in the duodenum. Both catheters were tunneled under the skin to the skull where they were fixed and connected to each other to maintain an intact enterohepatic circulation[15]. At 4 weeks after STZ injection, both catheters were disconnected and hourly bile samples were taken during 6 hours. Bile salt pool size and bile salt synthesis were determined from the wash-out curve as described[15].

Determination of secretory rate maximum of taurocholate (TC-SRm)

At 4 weeks after STZ or solvent injection, 6 control, 6 STZ and 6 STZ-Ins rats were anesthesized with pentobarbital (60 mg/kg BW). Catheters were placed in the common bile duct and in the jugular vein. Rats were kept in an incubator to maintain body temperature throughout the experiment. After 2 basal 10-minute bile collections, sodium taurocholate (TC; Calbiochem, La Jolla, CA) dissolved in 3% albumin/phosphate-buffered saline (PBS) (TC/PBS 2:1, TC concentration 100 mmol/l) was infused through the jugular catheter in stepwise increasing rates: 1, 2.5, 5, and 10 μmol/min/rat. The first three dosages were administered during 30 min periods, the last dose during 60 min. Bile was collected in 10 min intervals.

SRm was calculated as the mean of the 3 highest consecutive values of bile salt secretion and expressed as nmol/min/g liver.

Expression of hepatic transporter proteins

Four weeks after STZ or solvent injection, 6 control, 6 STZ and 6 STZ-Ins rats, were anesthesized with pentobarbital (60 mg/kg BW). Livers were rapidly excised and weighed. Parts of the liver were snap-frozen in liquid nitrogen for isolation of plasma membranes or messenger RNA (mRNA). For immunohistochemistry studies, small pieces of liver were frozen in isopentane and stored at -80°C until further use. Blood samples were collected in EDTA- and fluoride-EDTA containing tubes and centrifuged immediately (10,000g), and plasma was stored at -20°C until used.

Antibodies

Mouse monoclonal antibody P_3II-26, raised against the human MDR3[16], but also detecting rat Mdr2[17] was kindly provided by Dr. R.J. Scheper, Free University Hospital, Amsterdam, Netherlands. Polyclonal antibodies were raised against synthetic peptides of rat Bsep (k12) and Mrp2 (k4) as previously described[18]. Mouse monoclonal antibody against dipeptidyl peptidase IV (DppIV/CD26) was purchased from Endogen (Woburn, MA).

Steady state mRNA levels determined by reverse transcription polymerase chain reaction and real time quantitative polymerase chain reaction

Total RNA was isolated from frozen rat liver using TRIzol Reagent (GIBCO BRL, Grand Island, NY) according to the manufacturer's instructions. Single-stranded complementary DNA (cDNA) was synthesized from 4.5 μg RNA and subsequently subjected to reverse transcription polymerase chain reactions (RT-PCRs) as described by Vos et al[18]. Specific primer sets for the various ABC proteins have been described previously[18]. For every PCR reaction, β-*Actin* was used as the internal control. The number of cycles used was 25 for β-*Actin*, 24 for *Mrp2*, 27 for *Mdr2* and 24 for *Bsep*. In each experiment water was used as negative control. 7.5 μl of the PCR product was loaded on a 2.5% agarose gel and stained with ethidium bromide. Images were taken using a charge-coupled device video camera of the Image master VDS system, and density of individual bands was quantified using Image Master 1D elite v 3.00 software (Pharmacia, Uppsala, Sweden).

Real time quantitative PCR was performed on cDNA samples as described by Heid et al.[19] to detect mRNA levels of Sterol Regulatory Element Binding Protein1c (*SREBP1c*). *SREBP1c* primers were added and detection probes for *SREBP1c*, labeled with a fluorescent reporter dye (FAM) and a fluorescent quenching dye (TAMRA). Fluorescence was measured in each PCR tube for 25 msec and reexamined every 8.5 sec by an ABI Prism 7700 Sequence Detector v. 1.6 software

(Perkin-Elmer Corp., Foster City, CA). For every PCR reaction, *18S* was used as the internal control. In each experiment, water was used as negative control. The cycle number at the treshold (C_T), whereafter the intensity of reporter fluorescent emission increases, was used to quantitate the PCR product.

Isolation of liver plasma membranes

Liver plasma membrane fractions were isolated by the methods of Meier and Boyer[20], with modifications as detailed by Wolters et al[21]. Membrane fractions were stored at -80°C in 10 mmol/l Tris-HCl (pH 7.4) and 250 mM sucrose, supplemented with complete protease inhibitor cocktail (Boehringer Mannheim GmbH, Mannheim, Germany).

Western blot analysis

The protein concentrations in membrane fractions were determined as described by Lowry et al.[22], using bovine serum albumin as standard. Relative enrichment of Mg^{2+}adenosine triphosphatase (ATPase) as marker enzyme for the canalicular fraction was used to determine the degree of purification of the isolated membranes in the different experimental groups. Mg^{2+}ATPase activity was measured according to Scharschmidt et al[23]. About 15 µg of membrane proteins, corrected for MgATPase enrichment, was separated on 4%-15% Tris-HCl ready gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). For dipeptidylpeptidase IV (DppIV) detection, samples were boiled in sample buffer for 10 minutes before loading on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The blots were incubated with the first antibody diluted in Tris-buffered saline containing 5% dried milk powder (Fluka Bio Chemica, Buchs, Switzerland) and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma, St. Louis, MO), washed in Tris-buffered saline/0.1% Tween 20, incubated with horseradish peroxidase-labeled donkey anti-rabbit immunoglobulin (Ig) G or sheep anti-mouse IgG (dilution 1:1000; Amersham), and detected by the ECL Western blotting kit (Amersham).

Immunohistochemistry and confocal scanning laser microscopy

Four-micrometer sections were cut from frozen liver tissue and prepared for immunohistochemical studies as described by Hooiveld et al[17]. Confocal scanning laser microscopy (CSLM) was performed as described by Vos et al[18].

Analytical procedures

Bile salt concentrations in bile and plasma were determined by an enzymatic fluorimetric assay (Sterognost-Flu; Nyegaard & Co., Oslo, Norway). Levels of cholesterol and phospholipids were measured in bile as described[15]. Biliary bile salt composition was determined by capillary gas chromatography as described

by Wolthers et al.[24], after extraction of the bile salts from bile by use of Sep-Pack C_{18} cartridges (Waters Associates, Milford, MA). Plasma total bilirubin, alkaline phosphatase (AP), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH), γ-glutamyltranspeptidase (GGT) and glucose in plasma and bile were determined by routine clinical chemistry. Glutathione in liver and bile was determined as described by Griffith[25]. Biliary lactate and β-OH-butyrate were determined as described by Lawson et al[26].

Statistical analysis

All results are presented as means ± standard deviation (SD). Statistical analysis was performed using Kruskal-Wallis non-parametric analysis of variance, followed by Dunn's multiple comparisons test, or if 2 groups were compared, Mann-Whitney U test. As we used a non-parametric statistical test, it would theoretically be more appropriate to use median and range in stead of mean and SD. For reasons of clarity (big ranges) we used mean and SD. In calculating significance we used raw data, no mean and SD. Levels of significance for all statistical analysis was set at $p<0.05$.

RESULTS

Animal characteristics

STZ rats and STZ-Ins rats had a significantly lower body weight compared with control rats ($p<0.05$, Table 1). The ratio of liver to body weight was significantly increased in STZ- and STZ-Ins rats compared with controls ($p<0.05$, Table 1). Blood glucose concentrations were significantly higher in STZ-rats compared with controls, whereas those of STZ-Ins rats were intermediate (Table 1).

Table 2 shows plasma parameters of liver function in control, STZ- and STZ-Ins rats. In STZ rats, plasma bile salts, alkaline phosphatase (AP), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and γ-

Table 1: Characteristics of control, diabetic and insulin-treated diabetic rats.

	Body weight (g)	Liver weight (g)	Ratio of liver to body weight (%)	Plasma glucose (mmol/l)[b]
Control	396 ± 23	15.2 ± 0.9	3.8 ± 0.1	5.8 ± 0.3
STZ	280 ± 24[a]	14.4 ± 1.1	5.2 ± 0.3[a]	23.2 ± 3.5[a]
STZ + insulin	295 ± 26[a]	17.8 ± 4.1	6.1 ± 1.2[a]	11.9 ± 5.4

Measurements were performed at 4 weeks after induction of diabetes by STZ. Insulin treatment was given during the last 7 days before blood and tissue samples were collected. Data represent means ± SD, n = 6 per group.
a: $p<0.05$ compared with control group
b: glucose conversion factor from mmol/l to mg/dl: multiply by 18

Table 2: Plasma parameters of liver function in control, diabetic and insulin-treated diabetic rats.

	Control	STZ	STZ + insulin
Bile salts (μmol/l)	14.2 ± 7.5	44.8 ± 16.5[ab]	13.5 ± 5.7
Total bilirubin (μmol/l)	6.5 ± 2.3	10.2 ± 3.2	6.7 ± 1.4
AP (U/L)	30.2 ± 5.0	91.8 ± 34.5[a]	70.7 ± 20.7[a]
LDH (U/L)	539 ± 294	784 ± 410	863 ± 388
ASAT (U/L)	70.3 ± 11.4	225.3 ± 86.6[a]	162 ± 94.8
ALAT (U/L)	37.7 ± 5.4	178.2 ± 47.1[a]	107.3 ± 66.4[a]
GGT (U/L)	ND	3.0 ± 1.1[a]	1.3 ± 0.5

Data represent means ± SD, n=6 per group.
ND: not detectable
a: $p<0.05$ compared with controls
b: $p<0.05$ compared with STZ + insulin

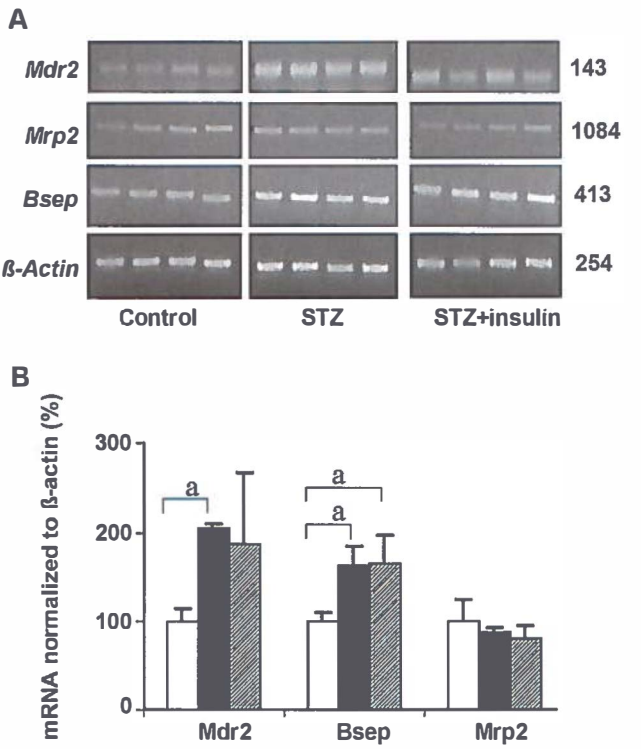

Figure 1. (**A**) *Mdr2*, *Bsep*, *Mrp2* and β-*Actin* mRNA expression in representative control, STZ and STZ-Ins rats. Total RNA was isolated from control, STZ and STZ-Ins rat liver, and 4.5 μg RNA was transcribed into cDNA and subjected to PCR analysis as described in Materials and Methods. Each band represents the results of a single animal. PCR products are indicated at the left side of each gel, corresponding base pairs at the right side, and the experimental group below the figure. (**B**) Bar diagram showing relative mRNA expression levels of *Mdr2*, *Bsep* and *Mrp2*, normalized to β-*Actin*, in control (open bars), STZ (closed bars), and STZ-Ins rats (hatched bars). n = 4, a: $p<0.05$ compared with controls.

glutamyltranspeptidase (GGT) concentrations were all significantly elevated compared to controls. The observed effects were partially reversed by insulin treatment.

Effects of STZ-induced diabetes on hepatic ABC transporter expression

The mRNA levels of selected hepatic transport proteins were analyzed by RT-PCR. Upon RT-PCR, mRNA levels of *Mdr2* (+105%) and *Bsep* (+60%) were significantly elevated in STZ rats compared with controls, when normalized for

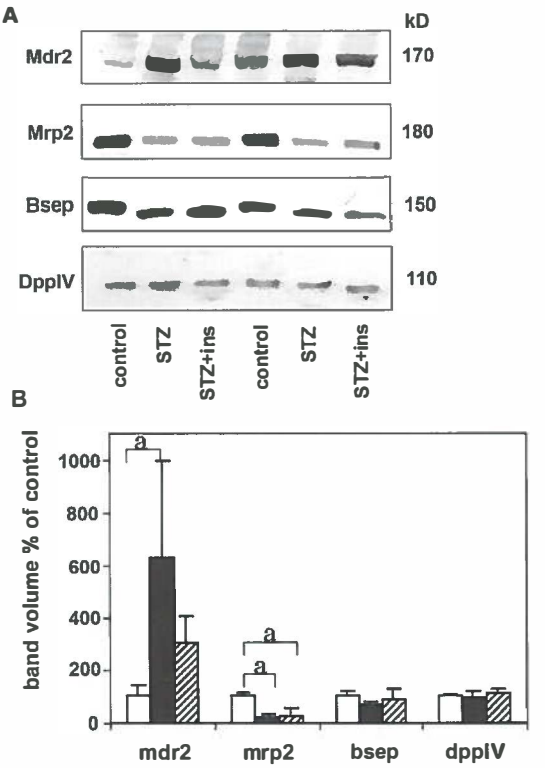

Figure 2. (**A**) Mdr2, Mrp2, Bsep and DppIV protein levels in plasma membrane fractions of control, STZ-diabetes (STZ) and STZ-Ins (STZ+ins) rat liver. Livers from control, STZ and STZ-Ins rats were used for isolation of plasma membranes; approximately 15 μg of membrane proteins, based on MgATPase enrichments (as described in Materials and Methods), was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. Immunoblotting was performed using the primary antibodies P_3II-26, k4, k12 and dppIV/CD26, recognizing Mdr2, Mrp2, Bsep and DppIV, respectively. Bound antibodies were visualized as described in Materials and Methods. Each band represents the results of a single animal. Transport proteins are indicated at the left side of each blot, apparent molecular weights at the right side and experimental group below the blot. Two representative experiments of 6 rats per group are shown. (**B**) Bar diagram showing western blot band volume as percent of control for Mdr2-Pgp, Mrp2, Bsep and DppIV, corrected for MgATPase enrichment in control (open bars), STZ (closed bars), and STZ-Ins rats (hatched bars). n = 6, a: $p<0.05$ compared with controls

β-*Actin* mRNA ($p<0.05$; Figure 1). In STZ-Ins rats, *Mdr2* mRNA levels tended to be higher compared with controls (not significant), whereas *Bsep* mRNA levels were significantly higher than in controls. No major changes were found in mRNA levels of *Mrp2*. As expected[27], mRNA levels of the insulin-controlled transcription factor *SREBP1c* were strongly reduced (-85%) in STZ rats compared with controls. In STZ-Ins rats, *SREBP1c* mRNA levels were still significantly lower (-60%) than in controls but higher than in STZ rats (data not shown).

Protein levels of the transporters were examined by Western blot analysis on liver plasma membranes (Figure 2). Protein levels of Mdr2-Pgp were 530% increased (±370% SD, n=6) in STZ-rats, compared with controls. In STZ-Ins rats, Mdr2-Pgp content returned towards control levels. Bsep protein levels were similar in control, STZ, and STZ-Ins rats. Both in STZ- and STZ-Ins rats, levels of Mrp2 were clearly decreased (-80% in STZ, -70% in STZ-Ins rats, respectively) compared with controls. Levels of DppIV, used as a canalicular marker protein, were similar in all 3 groups.

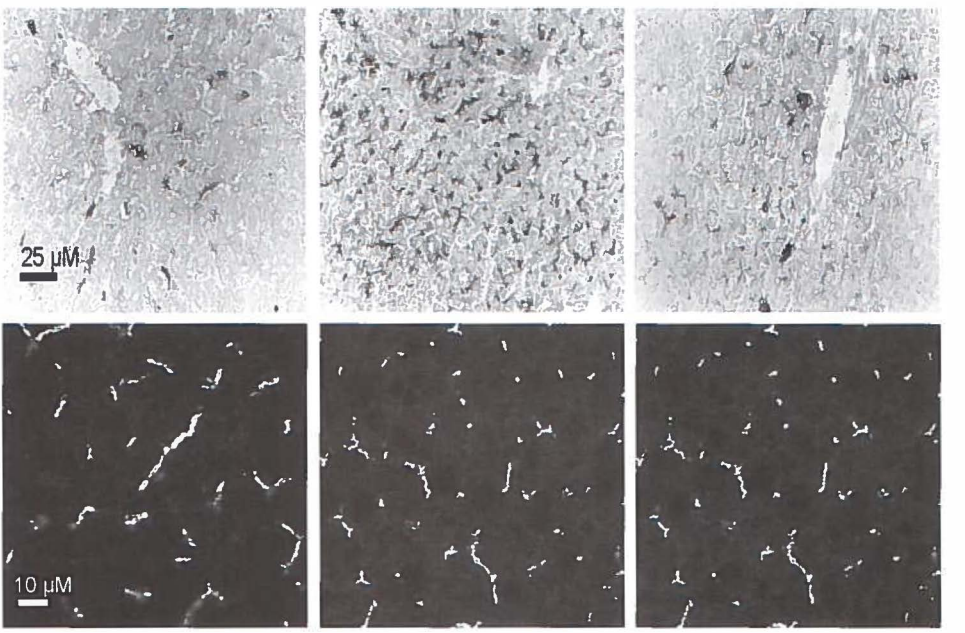

Figure 3. Immunohistochemical localization of (**A-C**) Mdr2-Pgp in (**A**) control, (**B**) STZ, and (**C**) STZ-Ins rats. Frozen liver sections were stained with primary antibodies directed against Mdr2-Pgp and Bsep using P_3II-26 and k12 antibodies, respectively. (**A**) In normal rat liver, staining of Mdr2-Pgp, although weak, was predominantly present in periportal regions. (**B**) In STZ rats, Mdr2-Pgp staining was strongly increased in periportal areas. (**C**) In STZ-Ins rats, the staining was comparable to controls. p, portal area. CSLM of (D) Bsep in control rats, (**E**) Bsep in STZ rats, and (**F**) Mdr2-Pgp in STZ rats showed localization of Bsep and Mdr2-Pgp exclusively at the hepatic canalicular membrane. In STZ rats the localization of Bsep and Mdr2-Pgp is unchanged compared with controls.

Localization of selected hepatic transport proteins was studied by immunohistochemistry and CSLM. In control liver, the intensity of P3II-26 signal (detecting mdr2 Pgp), was weak but stronger in periportal than in perivenous regions (Figure 3A). In livers of STZ rats, the signal was strongly increased in periportal areas (Figure 3B). In STZ-Ins rats the signal was comparable to that in controls (Figure 3C). In control liver, Bsep, as detected by k12, was uniformly distributed across the liver acinus; this pattern was not affected by induction of diabetes (data not shown). In CSLM studies Bsep and Mdr2-Pgp were localized at the hepatic canalicular membrane. In STZ rats the cellular localization of Bsep (Figure 3D and E) and Mdr2-Pgp (Figure 3F) was unchanged compared with controls.

Effects of STZ-induced diabetes on bile formation

In STZ rats, biliary concentrations of bile salts and phospholipids were increased compared with controls (Table 3). Basal biliary secretion rates of bile salts were increased by 260% in STZ rats compared with controls (Figure 4A), whereas basal biliary phospholipid output was increased by 520% (Figure 4B). Biliary glutathione concentrations were significantly decreased in STZ rats compared with controls (Table 3), and paralleled the relative amount of Mrp2 protein (Figure 5). Glutathione concentrations in the liver were also significantly decreased in diabetic rats compared with controls and STZ-Ins rats (27.1 ± 6.5, versus 46.0 ± 5.9 and 49.9 ± 18.1 nmol/mg, respectively; $p<0.05$ STZ compared with control and STZ-Ins). The biliary concentrations of glucose and β-hydroxy-butyrate were significantly increased in STZ rats compared with controls (Table 3).

In STZ rats, bile salt composition in basal bile samples showed an increased

Table 3: Basal bile flow and concentration of several bile constituents in control, diabetic and insulin-treated diabetic rats.

	Control	STZ	STZ +insulin
Bile flow (µl/min/g liver)	1.56 ± 0.25	1.90 ± 0.41	1.65 ± 0.36
Bile salt (mmol/l)	33.7 ± 4.6	98.1 ± 18.6[a]	57.7 ± 14.7
Phospholipid (mmol/l)	3.06 ± 0.27	15.8 ± 3.45[a]	6.97 ± 3.80
Cholesterol (mmol/l)	0.49 ± 0.10	0.51 ± 0.10	0.38 ± 0.16
PL/BS ratio	0.09 ± 0.02	0.16 ± 0.04	0.12 ± 0.04
Glutathione (mmol/l)	4.49 ± 0.99	0.13 ± 0.25[a]	0.79 ± 0.55[a]
Glucose (mmol/l)	0.32 ± 0.40	9.94 ± 3.41[a]	2.90 ± 5.43
Lactate (mmol/l)	2.51 ± 0.82	1.59 ± 0.65	2.25 ± 1.39
ß-OH-butyrate (mmol/l)	0.01 ± 0.02	1.23 ± 0.98[a]	0.30 ± 0.56

Bile was collected from pentobarbital-anesthesized animals for 20 minutes immediately after creation of a bile fistula. Data represent means ± SD, n = 5-6 per group
PL/BS ratio = Phospholipid to bile salt ratio
a: $p < 0.05$ compared to controls

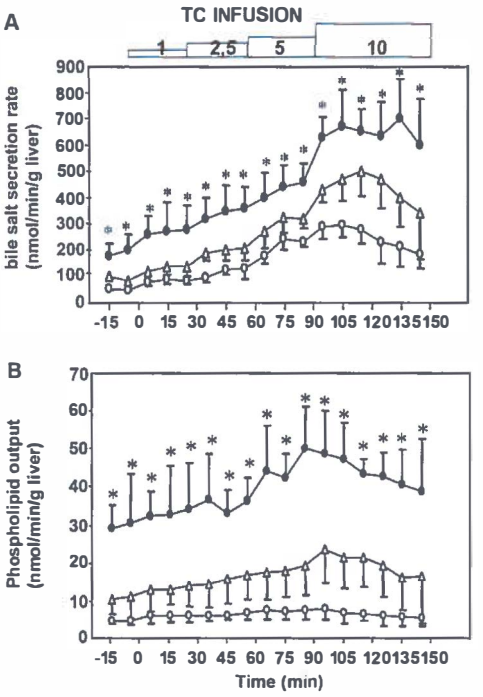

Figure 4. In control (open circles), STZ (closed circles), and STZ-Ins rats (open triangles), (A) bile salt secretion rate and (B) phospholipid output was determined during stepwise increasing TC infusion (n = 5 per group). At 4 weeks after STZ injection, rats were anesthesized with pentobarbital (60 mg/kg BW), and anesthesia was maintained by small doses of the drug during the experiment. Catheters were placed in the common bile duct and the jugular vein. To determine the TC-SRm, after two basal 10-min bile collection periods, TC dissolved in NaCl 0.9% (100 mmol/l) was infused through the jugular catheter at stepwise increasing rates: 1, 2.5, 5, 10 μmol/min/rat. The first three dosages were administered during 30-minute periods, the last dose during a 60-minute period. Bile was collected in 10-minute intervals. * $p < 0.05$, STZ significantly different from controls.

Figure 5. Mrp2 protein level and glutathione concentrations in bile in control, STZ, and STZ-Ins rats. Mrp2 protein level was determined by immunoblotting as described in Materials and Methods using the primary antibody k12. Western blot band volumes were expressed as percent of control. Glutathione concentrations in bile were determined in the basal bile samples taken by the SRm study and determined as described in Materials and Methods. Data represent the mean of 6 rats per group. a, $p < 0.05$ compared with controls.

proportion of cholate (93.3% of total bile salts) compared with controls (61.1%). Accordingly, concentrations of relatively hydrophilic bile salts like Δ22β-muricholate, α-muricholate, hyodeoxycholate, ursodeoxycholate were reduced in bile of STZ rats (Table 4).

Bile salt pool wash-out in unrestrained rats with STZ-induced diabetes and controls revealed that bile salt synthesis was increased by ~50 % and bile salt pool size by ~160 % in STZ rats compared with controls (data not shown).

Table 4: Relative basal biliary bile salt composition in control, diabetic and insulin-treated diabetic rats.

	Control	STZ	STZ +insulin
Cholate	61.1 ± 7.8	93.3 ± 2.5[a]	78.9 ± 6.9
Δ22ß-Muricholate	19.5 ± 5.5	2.0 ± 1.5[a]	9.9 ± 2.6
α-Muricholate	4.6 ± 0.5	0 ± 0a	2.0 ± 1.8
Chenodeoxycholate	5.0 ± 1.5	1.2 ± 0.8[a]	2.7 ± 1.0
Deoxycholate	3.7 ± 1.3	2.1 ± 0.9	3.1 ± 1.3
Hyodeoxycholate	2.8 ± 1.7	0.02 ± 0.05[a]	0.9 ± 1.3
Ursodeoxycholate	3.3 ± 1.5	1.5 ± 0.8[a]	2.5 ± 0.6

NOTE. Bile was collected from pentobarbital-anesthesized animals for 20 minutes immediately after creation of a bile fistula. Bile salt composition was analyzed by gaschromatography. Data represent means ± SD in percentage of total bile salt concentration, n = 5-6 per group.
a: $p < 0.05$ STZ compared with controls

Effects of STZ-induced diabetes on SRm of TC

To establish the effect of STZ on SRm of TC (TC-SRm), stepwise increasing doses of TC were infused intravenously in bile duct-canulated rats. Bile salt and phospholipid secretion rates were significantly higher in STZ rats compared with controls at every timepoint, whereas secretion rates of STZ-Ins rats were not significantly different from control values (Figure 4A and B). In STZ rats, the TC-SRm was increased by 130% compared with the SRm in controls ($p<0.01$). At SRm conditions, phospholipid to bile salt ratio was markedly higher in STZ rats than in control and STZ-Ins rats (Figure 6), in accordance with the increased Mdr2-Pgp content of hepatic plasma membranes in STZ rats (Figure 2). Figure 7 shows the correlation between bile salt secretion rate at TC-SRm and phospholipid concentrations determined in the same samples (mean of 3 consecutive phospholipid concentrations) in individual control, STZ and STZ-Ins rats. A significant positive relationship between the two parameters was found ($R=0.82$, $p<0.001$).

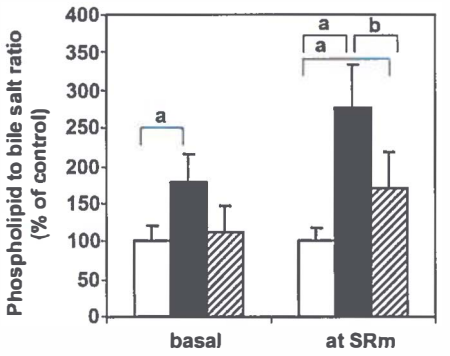

Figure 6. In control (open bars), STZ (closed bars), and STZ-Ins rats (hatched bars), phospholipid concentration to bile salt concentration ratios were determined at start of the TC-SRM study (basal) and at TC-SRM conditions (at SRm). TC-SRm was determined by stepwise increasing TC infusion as described in Materials and Methods. SRm was calculated as the mean of the 3 highest consecutive values of bile salt secretion. Bars represent the mean of 5 rats. a, $p < 0.05$ compared with controls; b, $p < 0.05$ compared with STZ-Ins rats.

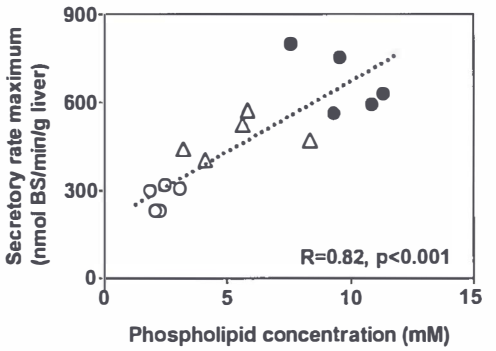

Figure 7. Bile salt secretion rate determined at the TC-SRm are compared with phospholipid concentrations determined in the same bile samples. TC-SRm was determined by stepwise increasing TC infusion as described in Materials and Methods. SRm was calculated as the mean of the three highest consecutive values of bile salt secretion. Points represent the TC-SRM per single rat. Control (open circles), STZ (closed circles), STZ-Ins rats (open triangles).

DISCUSSION

This study shows that STZ-diabetes in rats is associated with specific changes in the expression of canalicular ABC transporters that contribute to the characteristic diabetes-related alterations in bile formation[3]. Induction of *Mdr2* gene expression and Mdr2-Pgp content of liver plasma membranes appeared to represent a prominent consequence of STZ-diabetes that in all likelihood contributes to the high biliary phospholipid output associated with this condition[3]. In spite of the markedly elevated biliary bile salt output and the strongly increased TC-SRm, Bsep content of canalicular membranes isolated from diabetic rats was not increased. This finding implies that (1) "normal" Bsep levels are sufficient to accommodate the increased hepatic bile salt flux related to the diabetes-induced enlargement of the circulating bile salt pool, albeit in the face of slightly elevated plasma bile salt levels, and (2) Bsep protein expression is not the key determinant of bile salt SRm in rats as measured during supraphysiological bile salt infusion rates. The canalicular organic anion transporter Mrp2 was down-regulated in rats with

experimentally-induced type 1 diabetes by a post-transcriptional mechanism, which probably explains low biliary secretion rates of reduced glutathione equivalents[9]. The alterations in transporter expression and in bile formation were, at least in part, reversible by insulin treatment of diabetic rats, indicating that they were related to the absence of insulin per se and not to potentially toxic actions of STZ in the liver. Data on bile formation in uncontrolled diabetic patients support the concept that insulin deficiency per se alters the bile formation process[1].

STZ is widely used to induce insulin-deficiency in rodents, acting by destruction of pancreatic beta-cells through inhibition of beta-cell O-N-acetylglucosamine-selective N-acetyl-beta-D-glucosaminidase[28]. Because STZ exerts transient hepatotoxic actions[4,28], experiments were performed at 28 days after intraperitoneal injection of the drug, i.e., an experimental time frame similar to that described by others[3,4,29]. Plasma parameters of liver function, including bile salts and enzyme activities, were elevated in the diabetic rats in comparison to controls, and partially normalized in the insulin-treated diabetic rats. Insulin treatment of diabetic rats lowered plasma glucose levels, but these levels still tended to be higher than in controls (Table 1). This suggests that these rats were still partially insulin deficient, probably a result of the unphysiological mode of insulin administration. We speculate that the elevated liver function parameters are predominantly caused by insulin deficiency. This could result from an increased hepatic bile salt flux due to enlargement of the circulating bile salt pool and/or the relative shift towards more hydrophobic bile salt species. Changes in bile salt metabolism and bile formation similar to those observed in this study, i.e., increased biliary bile salt secretion, enlarged bile salt pool enriched in cholate, elevated bile salt biosynthesis, increased bile salt SRm and decreased biliary glutathione secretion, were reported previously in rats with uncontrolled diabetes caused by STZ[3,6,9] or alloxan treatment[5]. Increased bile salt synthesis in diabetic rats (~50% in the present study) is probably related to insulin-deficiency, because insulin is known to suppress expression of the key enzymes in bile salt synthesis, cholesterol 7α-hydroxylase and sterol 27-hydroxylase[8].

A key finding of this study is that expression of *Mdr2* is markedly induced in diabetic rats, resulting in a 530% increase in Mdr2-Pgp content of isolated liver plasma membrane fractions (Figure 2). Immunohistochemistry revealed that Mdr2-Pgp is predominantly periportally localized in diabetes (Figure 3B), i.e., it remained expressed in those cells of the liver lobule that accommodate most of the transhepatic bile salt flux during enterohepatic cycling.[30] Mdr2-Pgp is essential for biliary phospholipid secretion, probably by acting as a "flippase" that translocates bile-type phosphatidylcholines from the inner to the outer leaflet of the canalicular membrane[11]. Studies in Mdr2-Pgp-deficient mice[11] and transgenic mice overexpressing their human homologue MDR3[12] have shown that expression levels of this protein regulate the rate of bile salt-stimulated biliary phospholipid

secretion. Basal biliary phospholipid secretion was increased by 520% in STZ rats and tended to normalize during insulin treatment, in parallel with normalization of Mdr2-Pgp levels in the latter situation. Because phospholipid secretion is driven by bile salt secretion[31], (part of) this increase may be a result of the enhanced bile salt output. Yet, a specific Mdr2-Pgp-dependent effect is also clearly present as indicated by increased biliary phospholipid-to-bile salt ratios (+77% under basal conditions, +176% during maximal bile salt stimulation (Figure 6)). Recent studies have indicated a multifactorial regulation of *Mdr2* expression in rodents and several factors implied may be involved in its upregulation during diabetes. First, feeding of cholate, but not of hydrophilic ursodeoxycholate[32], has been shown to induce *Mdr2* expression in rat liver. Therefore the increased cholate pool size in STZ rats may induce *Mdr2* expression in this condition. Secondly, studies using fibrates[33], combined with the use of peroxisome proliferators-activated receptor α (PPARα)-deficient mice[34], have implicated a role of the nuclear receptor PPARα in control of *Mdr2* gene transcription. As fatty acids are natural ligands of PPARα[35], and insulin deficiency is associated with an increased flux of free fatty acids to the liver[36], the metabolic derangements of fatty acid metabolism that develop during diabetes may contribute to induction of *Mdr2* expression. Finally, recent studies from our laboratory have indicated a role for SREBPs in the regulation of *Mdr2* gene transcription[17]. SREBPs are comprised of a family of transcription factors that control various steps in cholesterol and fatty acid biosynthesis[37]. Recently, Shimomura et al.[27] showed that short-term insulin deficiency leads to a specific reduction in expression and nuclear protein levels of SREBP1c in rat liver without affecting those of SREBP1a. We were able to show a similar effect in livers of rats during long-term insulin deficiency, i.e., a reduction of *SREBP1c* mRNA by 85% without any change in *SREBP1a* mRNA levels (data not shown). Because SREBP1a is a particularly potent inductor of *Mdr2* promotor activity (Hooiveld G.J., Thesis, Groningen University 2000), a change in the ratio between 1a and 1c isoforms may contribute to enhanced *Mdr2* gene expression in diabetes. The exact role of insulin (deficiency) in the regulation of *Mdr2* expression is currently under investigation in our laboratory.

Bsep has been identified as the major canalicular transporter for bile salts[13]. Mutations in the human *BSEP* gene underlie progressive familial intrahepatic cholestasis type 2, a disease caused by the inability to secrete bile salts into bile as a result of absence of BSEP protein in the canalicular domain[38]. In view of the recently demonstrated regulation of *Bsep* gene expression by the "bile salt receptor" farnesoid X receptor[39], it was surprising to find that hepatic *Bsep* mRNA levels were only slightly increased upon induction of diabetes and that Bsep protein levels in plasma membrane fractions were unchanged, in spite of a 260 % increase in basal biliary bile salt output. On western blot, there seemed to be a slight shift in molecular weight of the Bsep bands in STZ treated livers. We speculate

that posttranslational modifications of Bsep protein in the diabetic state, such as altered glycosylation, could be the cause hereof. Immunohistochemistry and CSLM revealed that the homogenous distribution of the protein along the liver acinus as well as its localization to the canalicular membrane were maintained in diabetes (Figure 3D and E). Because basal biliary bile salt secretion rates were increased by 260% in STZ rats (Figure 4A), these findings implicate that this transport system under pathophysiological conditions has a considerable overcapacity.

The SRm has functionally been defined as the maximal amount of a certain bile salt that can be secreted into bile per unit of time before cholestasis is induced[40]. The SRm value for individual bile salt species appears to be related to their hydrophobicity[40] and shows marked interspecies variations[41]. A priori, we expected that the SRm would be related to the amounts of Bsep present at the canalicular membrane; however, this appeared not to be the case for TC. The SRm determined for TC in STZ rats was at least 130% higher than in controls, whereas Bsep levels were similar. Insulin-treatment of STZ rats reduced SRm values without affecting Bsep protein levels. Barnwell et al. proposed that cholestasis that occurs during infusion of supraphysiological amounts of bile salts reflects the detergent actions of biliary bile salts towards the canalicular membrane[42]. Biliary phospholipids are considered to protect the canalicular membrane against these actions by allowing the detergent molecules to be incorporated into mixed micelles[42]. Based on the observations of Barnwell et al.[42] that a drop in biliary phospholipids precedes the induction of cholestasis, these authors concluded that an inability to deliver sufficient phospholipids from their intracellular pool to the bile canalicular membrane leads to solubilization of the bile canalicular membrane by bile salts, as typified by the release of structural membrane phospholipids as sphingomyelin into bile, and leads to the development of cholestasis[42]. Our data show that the ability to secrete phospholipids into bile, a process governed by Mdr2-Pgp, is the actual determinant of the SRm of taurocholate in the rat (Figure 7). Thus, we propose that the maximal rate at which bile salts can be expelled into bile is not dictated by Bsep, but rather by Mdr2-Pgp function (Figure 8). Confirmation of the hypothesis that Mdr2-Pgp activity is a determinant of bile salt SRm could possibly be obtained by inducing diabetes in *Mdr2* knockout mice. However, *Mdr2* knockout mice develop a certain level of liver disease, which may confound interpretation of the results.

In contrast to protein levels of Mdr2-Pgp and Bsep, both members of the Abcb family of ABC transporters, levels of Mrp2 (Abcc2) were markedly reduced in STZ rats and remained depressed after insulin treatment (Figures 2 and 5). Down-regulation of Mrp2 protein expression involved a post-transcriptional event, because no changes in *Mrp2* mRNA levels were detected by RT-PCR (Figure 1). The mechanism underlying this effect of STZ-diabetes remains to be elucidated. Yet, it is highly likely that this effect is responsible for the reduction of bile salt-

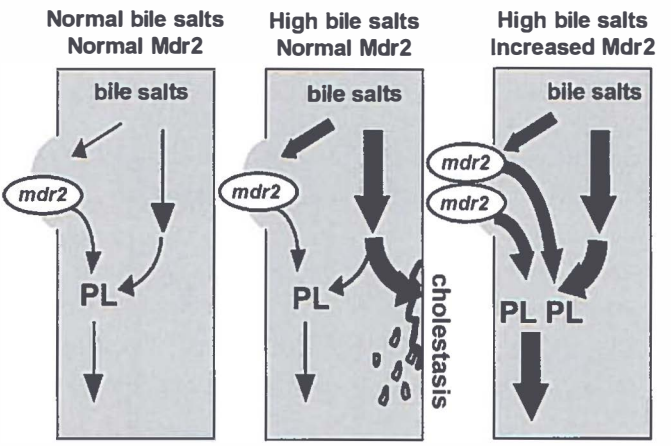

Figure 8. Hypothesized mechanism on the relationship between Mdr2-Pgp protein expression and bile salt SRm. In situations with "normal" Mdr2-Pgp levels and normal bile salt concentrations no cholestasis occurs, because sufficient phospholipid is available to shield the canalicular membrane from detergent bile salt actions. In situations with "normal" Mdr2-Pgp levels and high bile salt concentrations, bile salts within the canalicular lumen are not "detoxified" by phospholipids and cause cholestasis by disruption of canalicular membrane structure. With increased Mdr2-Pgp levels, high bile salt concentrations are compensated for by the high biliary phospholipid content and cholestasis will not be induced.

independent bile formation that has been reported previously in STZ-diabetic rats[9], as well as in spontaneously diabetic biobreeding rats[43]. Biliary glutathione has been identified as the major driving force for generation of the bile-salt-independent bile flow in rodents[44], and biliary secretion of glutathione appears to be mediated by Mrp2 because Mrp2-deficient rats lack biliary glutathione[14]. Direct evidence for involvement of Mrp2 in biliary glutathione secretion has recently been provided by Ito et al.[45] Lu et al.[46] have shown that the hepatic content and synthesis of glutathione is decreased in STZ-diabetic rats. Accordingly, we found a 40% decrease of hepatic glutathione content in livers of STZ rats, which normalized upon insulin administration. Treatment of STZ rats with insulin had only a slight effect in restoring Mrp2 expression and biliary glutathione concentrations (Figure 2 and 5).

In conclusion, increased biliary phospholipid secretion and decreased biliary glutathione secretion in STZ-diabetic rats coincide with increased protein expression of Mdr2-Pgp and decreased protein expression of Mrp2, respectively. Increased hepatobiliary bile salt flux and TC-SRm in STZ-diabetic rats are not associated with increased Bsep content of the canalicular membrane. We propose that Mdr2-Pgp activity controls maximal bile salt secretory capacity of the liver by providing "shielding capacity" in the form of phospholipids that protects the canalicular membrane from the detergent actions of bile salts.

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6

Enlarged bile salt pool in streptozotocin-diabetic rats associated with increased bile salt synthesis and enhanced bile salt reabsorption.

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Submitted

ABSTRACT

Background/Aims: Type 1 diabetes alters bile formation and bile salt metabolism. We evaluated the consequences of type 1 diabetes in rats on the kinetics of bile salt metabolism in relation to expression of the bile salt-activated nuclear receptor FXR.

Methods: Kinetics of bile salt metabolism were determined in vivo by isotope dilution of the bile salt pool with $^2H_4$-cholate in rats with streptozotocin-induced diabetes and in controls. Kinetic parameters were related to expression of genes involved in bile salt synthesis and transport.

Results: Diabetes increased cholate pool size and synthesis rate by 535 % and 106 %, respectively. Estimated daily intestinal cholate absorption was increased by 410 %. Diabetes did not affect hepatic mRNA levels of *Cyp7A1* or *Cyp 27* and increased that of *Cyp8B1*. *Fxr* expression was decreased by ~ 50% in diabetic rats. Surprisingly, expression of the FXR-target gene SHP, that mediates effects of FXR on bile salt synthesis genes, was unchanged in diabetes. Intestinal expression of the bile salt uptake protein *Asbt* appeared unaffected in diabetic rats.

Conclusions: Diabetes increases the cholate pool size in rats by stimulation of hepatic cholate synthesis, mainly via Cyp8B1, and by a concomitantly increased intestinal cholate reabsorption. Most likely, these effects are not mediated by altered bile salt signalling via FXR-SHP.

INTRODUCTION

Diabetes mellitus type 1 is a risk factor for development of atherosclerosis and is associated with hypercholesterolemia and hypertriglyceridemia[1,2]. The enterohepatic circulation of bile salts is a major regulator of cholesterol and triglyceride homeostasis and of plasma lipoprotein levels[3]. Diabetes mellitus is associated with an enlarged bile salt pool size in humans[4] and with an increased biliary bile salt excretion rate in experimental animals[5,6]. Bile salts facilitate the intestinal absorption of dietary fat, fat-soluble vitamins and cholesterol. On the other hand, the conversion of cholesterol into bile salts by the liver is quantitatively the most important pathway for removal of excess cholesterol from the body[3]. The majority of bile salts secreted by the liver into the intestine is reabsorbed and returns to the liver for resecretion into the bile. A relatively small fraction of bile salts is lost in the faeces and this loss equals *de novo* synthesis in the liver under steady state conditions[7]. An enlarged bile salt pool and an increased bile salt synthesis have been described in (experimental) diabetes[5,8], but there are no in vivo data on bile salt turnover and reabsorption.

Cholesterol 7α-hydroxylase (Cyp7A1), sterol 27 hydroxylase (Cyp27) and sterol 12α-hydroxylase (Cyp8B1) are key enzymes in bile salt synthesis. The Farnesoid X receptor (FXR) is a bile salt-activated nuclear receptor involved in feedback inhibition of bile salt synthesis. Activation of FXR by bile salts inhibits transcription of, amongst others, genes encoding *Cyp7A1* and *Cyp8B1*[9,10,11]. This effect of FXR is indirect: after heterodimerisation with the retinoid X receptor (RXR), activated FXR stimulates transcription of the small heterodimer partner (SHP). SHP subsequently dissociates the liver receptor homologue (LRH-1) from the promoters of *Cyp7A1* and *Cyp8B1*, which decreases transcription of these genes[9,10,11]. FXR-SHP-independent pathways for the suppression of *Cyp7A1* and *Cyp8B1* expression by bile salts have also been described[12,13]. It was shown recently that expression of the *Fxr* gene in rat liver cells is influenced by intracellular glucose (metabolites) and is strongly reduced in livers of diabetic rat models[14]. Consequently, reduced *Fxr* expression may contribute to enhanced bile salt synthesis in type 1 diabetes.

The aim of the present study was to establish the consequences of type 1 diabetes on the kinetics of bile salt metabolism and their relationship with hepatic expression of the bile salt-activated nuclear receptor FXR. For this purpose, a stable isotope dilution technique using $^2H_4$-cholate was employed in rats with streptozotocin (STZ)-induced diabetes, control rats and insulin-treated STZ-rats (STZ-ins rats). Kinetic parameters were related to the expression of relevant FXR-controlled genes in liver and intestine. Our results show that streptozotocin-induced diabetes in rats is associated with a pronounced increase in cholate pool size and synthesis rate as well as with enhanced intestinal cholate reabsorption, which are, in view of unaltered expression of several FXR-target genes, in all

likelihood independent from the diabetes-associated reduced FXR expression.

MATERIALS AND METHODS

Animals

Male Wistar rats (310-345 g) were purchased from Harlan (Zeist, the Netherlands) and housed in a temperature-controlled environment with alternating 12-hour light and dark. The rats received standard laboratory chow (RMH-B; Hope Farms BV, Woerden, the Netherlands) and had free access to food and water. Experimental procedures were approved by the local Ethics Committee for Animal Experimentation.

Experimental procedures

Two independent experiments were performed. In both experiments, diabetes was induced by a single intraperitoneal injection (6 mg/100 g body weight, volume of injection ± 0.2 ml) of streptozotocin (Pharmacia & Upjohn, Kalamazoo, Michigan, USA). Control animals received an intraperitoneal injection of the solvent (3% sodium citrate). Induction of diabetes was perceived by development of hyperphagia, polydipsia and polyuria and confirmed by determination of the degree of hyperglycemia.

Kinetics of bile salt metbolism

At day 24 after the STZ injection, all rats in this experiment were equipped with a permanent heartcatheter under halothane anaesthesia as described previously[15]. In the same session in half of the diabetic rats an insulin pellet was implanted subcutaneously (bovine insulin, insulin release about 2U/24 h during 40 days, Linplant, Linshin Canada Inc., Scarborough, Ontario, Canada). At day 30, [2,2,4,4-$^{2}H_{4}$]-cholate ($^{2}H_{4}$-cholate, isotopic purity 98%, Isotec, Miamisburg, OH) in a dosage of 5 mg/rat was administered intravenously. Subsequently, blood samples (0.3 ml) were obtained at 0, 6, 12, 24, 36, 48, 72 hours after administration of $^{2}H_{4}$-cholate. Plasma was obtained by centrifugation at 4000 rpm for 10 minutes and stored at -20 ºC until analysis.

Determination of bile flow, biliary and faecal bile salt excretion and hepatic and intestinal transcription factors/transporters

At four weeks after STZ or solvent injection, 6 control, 6 STZ and 6 STZ-Ins rats were anaesthesized with pentobarbital (60 mg/kg BW). The STZ-ins rats were treated with insulin for 1 week as described elsewhere[16]. Bile was collected for 20 min after cannulation of the common bile duct. The livers were rapidly excised and weighed. Parts of the liver were snap-frozen in liquid nitrogen for RNA isolation. Small intestines were flushed with a buffered salt solution; representative parts of

the intestine were snap-frozen in liquid nitrogen for RNA isolation or for Western blot analysis. The terminal part of the intestine was weighed, whereby the ileum was defined as the 45 cm proximal to the ileocecal valve. To measure faecal bile salt output, faeces was collected during 4 days before the end of the experiment. Blood samples were collected and centrifuged immediately (4000 rpm), and plasma was stored at -20°C until used.

Analytical procedures

Total bile salt concentration in bile was measured by an enzymatic fluorometric assay using 3α-hydroxysteroid dehydrogenase[17]. 1 μl bile was diluted in demineralised water and assayed. Biliary bile salt composition was determined by gas chromatography[18]. Faeces were homogenized and freeze dried. Dried faeces (25 mg) were treated with 1 mL alkaline methanol (methanol : 1 mol/L NaOH 3:1 (v/v)) for 2 h at 80°C in screw capped tubes. Then 9 mL of demineralised water was added and the tubes were mixed and centrifuged. Total bile salt concentration was determined on 100 μL of the supernatant with enzymatic/fluorimetric assay as described for bile[17]. The total residual supernatant was subsequently applied to a prepared Sep-Pak C18 solid phase extraction cartridge for determination of the bile acid composition by gas chromatography[18]. Plasma glucose was measured by routine clinical chemistry. HbA_{1c} was determined by ion-exchange high performance liquid chromatography (VARIANT™ HbA_{1c} Program with Bio-Rad VARIANT Hemoglobin Testing System, Bio-Rad, Hercules, CA). Plasma bile salt isotope analysis was performed by gas chromatography mass spectrometry (GC-MS) as described by Hulzebos et al[19].

RNA isolation and RT-PCR procedures

RNA isolation and cDNA synthesis were performed according to Bloks *et al.*[20]. Real time quantitative PCR was performed as described by Heid *et al.*[21], modified in our laboratory as described[22]. Primer and probe sequences (Invitrogen, Carlsbad, USA) and detection probes (Eurogentec, Seraing, Belgium) for the genes of interest, labeled with the 5' linked fluorescent reporter dye 6-carboxy-fluorescein (FAM) and the 3'linked fluorescent quenching dye 6-carboxy-tetramethyl-rhodamine (TAMRA), have been published[22,23]. Primers and probes of *Cyp8b1*, *Lxr*-α and *Shp* were as follows: *Cyp8B1* (NM_031241, forward AAG GCT GGC TTC CTG AGC TT, reverse AAC AGC TCA TCG GCC TCA TC, probe CCT GCT CCT TGT CCT TGG TGC AGC); *Lxr*-α (NM_031627, forward GCT CTG CTC ATA GCC ATC AG, reverse CAG GGC CTC CAC ATA TGT GT, probe TCT GCA GAC CGG CCC AAC GTG); *Shp* (NM_057133, forward ACC TGC AAC AGG AGG CTC ACT, reverse TGG AAG CCA TGA GGA GGA TTC, probe TCC TGG AGC CCT GGT ACC CAG CTA GC). Measurements were performed using an ABI Prism 7700 Sequence Detector with 1.6.3 software (Perkin-Elmer Corp., Foster City, California).

Western blotting

Homogenates of the intestine were made as described by Schmitz et al[24]. Total protein concentration of the homogenates was determined as described by Lowry et al[25]. Proteins (2.5 µg protein / lane) were separated on 4-15 % Tris-HCl ready gels (Bio-Rad Laboratories, Hercules, CA) and blotted onto nitrocellulose membranes by tank blotting. Asbt and Ibabp content were determined as described by Kok et al[26].

Calculations

The isotope dilution technique was performed as described by Hulzebos et al[19]. Enrichment was defined as the increase of M_4-Cholate / M_0-Cholate after adminstration of $^2H_4$-Cholate and expressed as the natural logarithm of atom % excess (ln APE) value. The cholate fractional turnover rate (FTR), pool size, synthesis rate, cycling time and loss per enterohepatic cycle were calculated[19,23]. The biliary bile salt and biliary cholate excretion rate were calculated by multiplying the bile flow (µl/h/rat) with respectively the biliary bile salt concentration (mM) or the biliary cholate concentration (mM). Faecal bile salt excretion rate was calculated by multiplying daily faecal dry weight with the faecal bile salt concentration (µmol/ gram dry weight). The faecal excretion of individual bile salts was calculated as percentage of total bile salt excretion.

Statistical analyses

Results are presented as means ± standard deviation (SD). Statistical analyses was performed using Kruskal-Wallis non-parametric ANOVA, followed by Mann-Whitney U test with Bonferroni correction, or if 2 groups were compared, Mann-Whitney U test. Level of significance for all statistical analyses was set at $p<0.05$.

RESULTS

Animal characteristics

Table 1 summarizes relevant characteristics of the animals used in the experiments. STZ rats and STZ-ins rats had significantly lower body weights than control rats, as previously described[16]. The ratio of liver to body weight was significantly increased in STZ- and STZ-ins rats compared to controls. Plasma glucose concentrations and blood HbA_{1c} percentages were significantly higher in STZ-rats than in controls and were intermediate in STZ-ins rats.

Effects of STZ-induced diabetes on biliary bile salt secretion and faecal bile salt excretion.

Bile flow was comparable in the 3 groups (Table 2). Biliary bile salt secretion rate and cholate secretion rate were significantly increased in STZ-rats compared with

Table 1: Characteristics of control, diabetic and insulin-treated diabetic rats

	Body weight (g)	Liver weight (g)	Ratio liver to body weight (%)	Plasma glucose (mmol/l)[c]	HbA1c (%)[d]
Control	395.6 ± 22.6	14.8 ± 1.2	3.8 ± 0.3	5.9 ± 0.3	1.8 ± 0.1
STZ	278.6 ± 20.1[ab]	14.5 ± 1.0[b]	5.2 ± 0.3[a]	24.4 ± 3.6[ab]	4.1 ± 0.2[ab]
STZ -ins	317.8 ± 32.3[a]	18.5 ± 3.3[a]	5.8 ± 0.9[a]	11.5 ± 5.8[a]	2.9 ± 0.4[a]

Measurements were performed at 4 weeks after induction of diabetes by STZ. Experimental details were described in the Materials and Method section. Data represent means ± SD, n = 11-13 per group.
a: $p < 0.05$ compared with control group
b: $p < 0.05$ compared with STZ-ins
c: glucose conversion factor from mmol/l to mg/dl: multiply by 18
d: HbA1c was measured in experiment 1 (n = 5-6 per group)

controls and STZ-ins rats (Table 2). Insulin treatment partly normalized bile salt- and cholate secretion rates (Table 2).

Daily faecal total bile salt excretion was increased by ~56 % in STZ-rats compared with controls (Figure 1). Faecal bile salts in STZ-rats consisted mainly of cholate and its major metabolite deoxycholate, whereas the proportion of chenodeoxycholate metabolites (iso-lithocholate, hyodeoxycholate and β-muricholate) was reduced ($P<0.05$, Figure 1B). Insulin treatment tended to normalize faecal bile salt output and composition.

Table 2: Bile flow, biliary bile salt concentration and bile salt secretion rates in control, diabetic and insulin-treated diabetic rats

	Control	STZ	STZ-ins
Bile flow (ml/h/rat)	1.5 ± 0.2	1.7 ± 0.4	1.7 ± 0.3
Bile salt concentration (mM)	33.7 ± 4.4	98.1 ± 16.6[ab]	57.7 ± 13.8[a]
Bile salt secretion rate (µmol/h/rat)	51.2 ± 2.4	161.2 ± 45.1[ab]	96.7 ± 12.5[a]
Cholate secretion rate (µmol/h/rat)	30.0 ± 2.1	149.5 ± 37.6[ab]	76.9 ± 16.1[a]

Bile was collected from pentobarbital-anaesthesized animals for 20 minutes immediately after creation of a bile fistula. Data represent means ± SD, n = 5 per group
a: $p < 0.05$ compared with control group
b: $p < 0.05$ compared with STZ-ins

Effects of STZ-induced diabetes on the kinetics of cholate metabolism

To gain insight in the underlying mechanisms of altered bile salt metabolism in STZ-diabetes, kinetics of the enterohepatic circulation of cholate were assessed. Figure 2 shows the decay of cholate enrichment in plasma after i.v. injection of $^2H_4$-cholate. From these data cholate pool size and synthesis rate could be calculated[19]. The cholate pool size was increased by 535 % in STZ-rats compared with controls (Table 3). Cholate pool size showed intermediate values in STZ-ins rats. The increased pool size in STZ-diabetes was associated with a strongly increased cholate synthesis rate (+ 106 %). The fractional turnover rate of

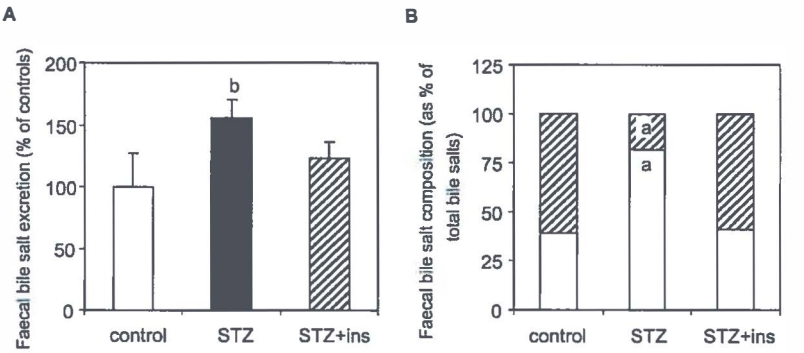

Figure 1. Faecal bile salt excretion and faecal bile salt composition in control-, STZ- and STZ-ins rats. Faeces were collected during 4 days before the end of the experiment. Faecal bile salt concentration and bile salt composition were determined as described in Materials and Methods. (**A**): Faecal bile salt excretion as % of control rats. In control rats faecal bile salt excretion was 16.0 ± 4.3 µmol/day. Data are means ± standard deviation of n = 5-6 per group. b: $p < 0.05$ compared to controls (**B**): Faecal bile salt composition as % of total bile salts. Open part of the bars is cholate + deoxycholate. Hatched part of the bars are chenodeoxycholate metabolites (isolithocholate, hyodeoxycholate and β-muricholate). a: $p < 0.05$ compared to controls and STZ-ins rats.

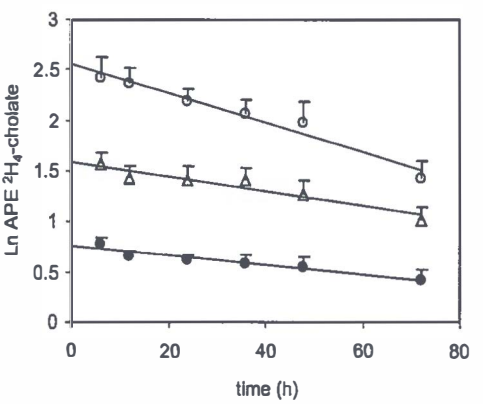

Figure 2. Kinetics of bile salt metabolism, studied by stable isotope dilution with $^2H_4$-cholate. Decay of intravenously administered $^2H_4$-cholate (5 mg/rat) in control (open circles), STZ- (closed circles) and STZ-ins rats (open triangles). Data are means ± standard deviation of n = 5 per group. Decay of $^2H_4$-cholate in time could be expressed with the following equations. In controls: [Ln APE $^2H_4$-cholate] = -0.0144[time] + 2.55 (R^2=0.96). In STZ: [Ln APE $^2H_4$-cholate] = -0.0046[time] + 0.75 (R^2=0.92). In STZ-ins: [Ln APE $^2H_4$-cholate] = -0.0072[time] + 1.59 (R^2=0.92).

Table 3: Cholate kinetic parameters obtained by 2H4-Cholate isotope enrichment measurements in plasma of control, diabetic and insulin-treated diabetic rats

	Control	STZ	STZ-Ins
Cholate pool size (µmol/rat)	85.2 ± 19.8	541 ± 18.7[ab]	237.5 ± 25.6[a]
Cholate synthesis rate (µmol/rat/day)	27.7 ± 5.5	57.2 ± 24.1[a]	41.5 ± 8.5
Cholate FTR (pools/day)	0.34 ± 0.11	0.11 ± 0.05[a]	0.18 ± 0.03[a]
Bile salt pool size (µmol/rat)	145.3 ± 30.0	580.6 ± 29.2[ab]	304.0 ± 50.7[a]

Data represent means ± SD, n = 5-6 per group
a: $p < 0.05$ compared to controls
b: $p < 0.05$ compared with STZ-ins

cholate in STZ-rats was decreased by 68 %. Total bile salt pool size, calculated from cholate pool size and percentage cholate in bile, was increased by 300% in STZ-rats (Table 3). The amount of cholate lost per enterohepatic cycle was estimated by dividing cholate synthesis rate by cholate cycling frequency[23] and was significantly increased in STZ-rats (Table 4). However, as the cholate pool was strongly increased in STZ-diabetes, the cholate loss per enterohepatic cycle, as fraction of the cholate pool, was smaller in STZ-rats compared to controls, namely 1.8 versus 3.8 %. From these values we estimated an increase in absolute cholate absorption by 410 % in STZ rats (Table 4).

Table 4: Estimation of bile salt pool size, cycling time and intestinal absorption in control, diabetic and insulin-treated diabetic rats

	Control	STZ	STZ-Ins
Cholate cycling time (h)	2.8 ± 0.6	3.8 ± 0.8	3.2 ± 0.9
Amount cholate lost per enterohepatic cycle (µmol)	3.3 ± 0.8	9.6 ± 5.6[a]	5.5 ± 1.3
Cholate absorption per enterohepatic cycle (% of cholate pool)	96.2 ± 0.6	98.2 ± 1.1[a]	97.7 ± 0.3[a]
Cholate absorption (µmol/rat/day)	691.7 ± 45.4	3532.0 ± 923.2[ab]	1803.7 ± 379.5[a]

Data represent means ± SD, n = 5-6 per group
a: $p < 0.05$ compared to controls
b: $p < 0.05$ compared with STZ-ins

Effects of STZ-induced diabetes on the expression of hepatic enzymes and transcription factors involved in bile salt synthesis

Figure 3A shows the effects of STZ-diabetes on hepatic mRNA expression of three enzymes involved in bile salt synthesis. The mRNA levels were normalized to β-actin. The expression of β-actin was unchanged under STZ or STZ-ins conditions. The mRNA levels of *Cyp7A1* were not affected in STZ-diabetes. *Cyp8B1* expression was strongly induced in STZ-diabetes. In the insulin-treated STZ-rats mRNA expression of *Cyp7A1* was comparable to controls, but mRNA levels of *Cyp8B1* remained significantly higher compared with control rats. The mRNA levels of *Cyp27* were not affected in either condition.

As shown previously[14], *Fxr* expression was decreased by ~ 50% in STZ-diabetic rats compared to controls and STZ-ins rats. However, the expression of *Shp* was comparable between the 3 groups (Figure 3B). In STZ-rats, mRNA levels of *Rxr* were reduced by 30 % compared to controls (Figure 3B). The mRNA levels of *Lxr* (liver X-receptor) were similar in STZ-rats and controls and were increased by ~ 25% in STZ-ins rats (Figure 3B).

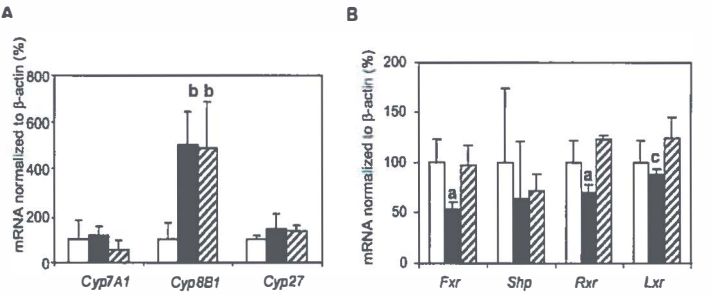

Figure 3. Changes in relative hepatic gene expression of *Cyp7A1*, *Cyp8B1*, *Cyp27*, *Fxr*, *Shp*, *Rxr* and *Lxr* upon induction of streptozotocin-diabetes in rats determined by realtime PCR. RNA was isolated from ~ 30 mg liver tissue from rats of the three groups and converted to cDNA. Levels of cDNA were measured by real-time PCR as described in the Method section. Results were normalized to β-*Actin* mRNA levels. Open bars, control rats; closed bars, STZ-rats; hatched bars, STZ-ins rats. Cyp7A1, gene encoding cholesterol 7 α-hydroxylase; Cyp8B1, 12 α-hydroxylase; Cyp27, sterol 27 hydroxylase; Fxr, farnesoid X receptor; Shp, small heterodimer partner; Rxr, retinoid X receptor; Lxr, liver X receptor. Data are means ± standard deviation of 4 rats per group, a: $p < 0.05$ compared with controls and STZ-ins rats; b: $p < 0.05$ compared with controls; c: $p < 0.05$ compared with STZ-ins rats

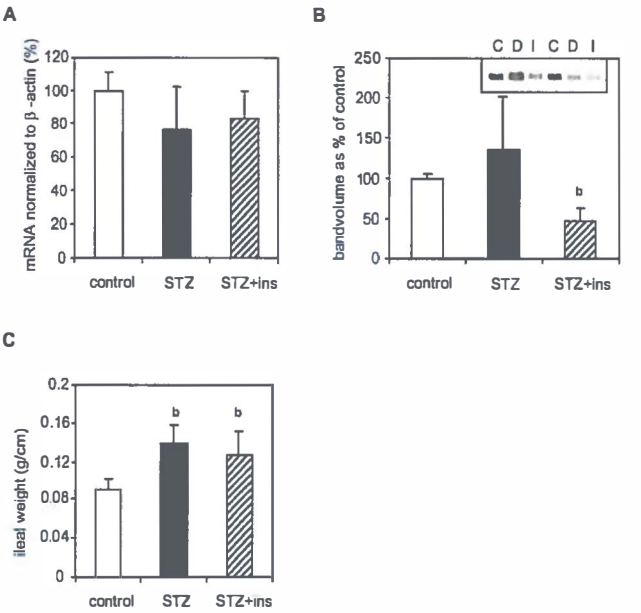

Figure 4. Changes in relative intestinal gene expression of *Asbt*, determined by realtime PCR, intestinal Asbt protein levels and ileal weight upon induction of streptozotocin-diabetes in rats. (**A**): RNA was isolated from ~30 mg intestinal tissue and processed essentially as described in the Legends of Figure 3. Data are means ± standard deviation of 4 rats per group. (**B**): Asbt protein levels in intestinal homogenates of the terminal ileum of rats of the three groups, isolated as described in the Methods section. Data are means ± standard deviation of 4 rats per group. C, control; D, STZ-rats; I, STZ-ins rats. (**C**): Ileal weight per cm, whereby the ileum was defined as the 45 cm proximal to the ileocecal valve. Data are means ± standard deviation of 6 rats per group. b: $p < 0.05$ compared with controls

Effects of STZ-induced diabetes on the expression of intestinal Fxr, and transporters involved in bile salt reabsorption, and on ileal weight

The expression of the main intestinal uptake system for bile salts, i.e., Asbt (apical sodium-dependant bile salt transporter) was not altered in STZ diabetes on mRNA (Figure 4A) and protein level (Figure 4B). Asbt protein levels in insulin-treated diabetic rats were significantly lower compared to controls. The ileal weight was increased by ~ 50% in STZ diabetes and by ~ 40% in STZ-ins rats (Figure 4C) compared with controls. In the diabetic state, we did not find an upregulation of Asbt protein levels in more proximal ileal enterocytes (data not shown). Ibabp (ileal bile acid binding protein) protein levels and intestinal *Fxr* gene expression were not significantly different between the three groups (data not shown).

DISCUSSION

This study demonstrates that STZ-induced type 1 diabetes in rats is associated with specific changes in the kinetics of bile salt enterohepatic circulation. We found both an increased cholate pool size and hepatic cholate synthesis in diabetes. From these data we could calculate that the efficiency of intestinal cholate absorption was concomitantly enhanced. These findings imply that the increased cholate absorption in diabetic rats did not lead to efficient repression of bile salt synthesis, as for instance is the case during bile salt feeding[27,28,29]. The high rate of cholate synthesis in STZ-diabetes was associated with increased gene expression of *Cyp8B1*. Yet, diabetes-induced reduction of FXR expression seemed not to be responsible for the enhanced bile salt synthesis in type 1 diabetes, since expected changes in FXR-target gene expression were not observed.

STZ is widely used to induce insulin-deficiency in rodents, acting by destruction of pancreatic beta cells through inhibition of beta-cell O-N-acetylglucosamine-selective N-acetyl-beta-D-glucosaminidase[30]. Since STZ exerts transient hepatotoxic actions[6,30] experiments were performed at 28-30 days after intraperitoneal injection of the drug, i.e., an experimental time frame similar to that described by others[5,6,31]. Alterations in expression of transcription factors and of cholate kinetics were, in part, reversible by insulin treatment, indicating that they were related to the absence of insulin *per se* and not to potentially toxic actions of STZ in liver or intestine. Available data on bile formation in uncontrolled diabetic patients support the concept that insulin deficiency *per se* alters the bile formation process[4].

We established the consequences of diabetes on kinetic parameters of cholate metabolism using a novel isotope dilution technique, applicable *in vivo* in unanaesthesized animals[19]. With this technique, cholate pool size, FTR and synthesis rate were determined simultaneously without experimental interruption of the enterohepatic circulation. In the representation of our kinetic results we

chose to express the parameters per rat and not per kg body weight, because STZ-rats had much lower body weights than controls, while liver and intestinal weights were higher. In STZ-rats we found an increased cholate pool size and synthesis rate, as described by others applying alternative techniques[8,32]. The estimated[23] cholate absorption per enterohepatic cycle (as percentage of the cholate pool size) increased from 96.2 % in controls to 98.2 % in STZ-rats (Tabel 4). The cholate absorption per day appeared to be increased by 410 % in STZ rats (Table 4). This implies that the enlarged cholate pool size in diabetes is associated with both an increased synthesis rate and a more efficient intestinal absorption. In STZ-ins rats, cholate kinetic data shifted towards control values but cholate pool size and absorption were still increased.

In STZ-diabetes, biliary bile salt secretion rate was significantly increased compared to controls while bile flow was unchanged, most likely as a consequence of reduction of the bile salt-independent bile flow together with an increase in bile salt-dependent bile flow. The decreased bile salt-independent bile flow has been reported previously in STZ-diabetic rats[33] as well as in spontaneously diabetic biobreeding rats[34] and is associated with down-regulation of multidrug resistance-associated protein 2 (Mrp2=Abcc2) protein expression[16]. We described previously the effects of STZ-diabetes on genes involved in bile formation[16].

The 50% increase in total bile salt synthesis in STZ diabetes was not associated with an increase in hepatic *Cyp7A1* mRNA expression (Figure3). An enlarged bile salt pool size would be expected to lead to suppression of bile salt synthesis via FXR-SHP[9,10]. In diabetic rats, hepatic *Fxr* mRNA levels were decreased, which may have been caused by prevailing low intracellular glucose concentrations[14]. When reduced *Fxr* mRNA levels would be the cause of the increased bile salt synthesis, we would have expected increased *Shp* mRNA and *Cyp7A1* mRNA levels. However, both were not significantly changed in the diabetic state. The increase in total bile salt synthesis (+50%) in rats with STZ-diabetes is therefore likely attributable to Cyp7A1 activity regulation at a post-transcriptional level and not associated with the decreased *Fxr* expression. Other authors did show a small increase in *Cyp7A1* mRNA in STZ-diabetes[14,35]. Since Cyp7A1 expression shows a marked day-night variation with the highest mRNA around 22.00hr and the lowest around 10.00hr, it cannot be excluded that mRNA expression was higher during the night in diabetics. Ishida et al, however, found no changes in Cyp7A1 activity in STZ-diabetes[35].

In the STZ-diabetic rats *Cyp8B1* expression was strongly increased, corresponding with the 106% increase in cholate synthesis in the diabetic state (Figure3, Table3). The enhanced *Cyp8B1* expression may have contributed to a reduction in chenodeoxycholate synthesis in the STZ-rats, as confirmed by the reduced fraction of muricholates in faeces (Figure 1). We described previously an increased cholate / chenodeoxycholate derivates ratio in bile of STZ-diabetic rats[16] and now also in faeces (Figure 1). Transcription of *Cyp8B1* is inhibited by

bile salts via FXR and SHP[10,11,36] and by insulin[35,37-39] similar to *Cyp7A1*. In contrast to *Cyp7A1*, it appears that *Cyp8B1* expression is stimulated by PPARα[40]. Increased PPARα activity due to increased fatty acid fluxes in the diabetic state could be a crucial factor in the observed selective stimulation of *Cyp8B1* expression. Ishida et al.[35] also found increased *Cyp8B1* mRNA levels in STZ-diabetes and an increased Cyp8B1 activity compared to controls. It is puzzling why the decreased FXR levels do not seem to be of influence. Other hepatic target genes of FXR are *Ntcp* (repressed gene)[41], *Bsep* and *Mrp2* (induced genes)[42,43]. As we showed earlier, STZ-diabetes is associated with unchanged *Ntcp* and *Mrp2* mRNA expression and increased *Bsep* mRNA levels[16], i.e., findings not at all corresponding with the decreased FXR expression in STZ-diabetes. Whether FXR signalling could be impaired by the high concentrations of free fatty acids and ketone bodies in the diabetic state, as described earlier for LXRα[44,45,46], has to be further investigated.

The enhanced cholate absorption in STZ-diabetic and insulin-treated STZ rats may be the result of an enlarged intestinal absorptive area and, thereby, an increased number of Asbt transporter proteins. Bile salts are absorbed from the distal ileum via the transporter Asbt[47]. Ibabp, a 14 kD cytosolic protein, has been suggested to be involved in bile salt reabsorption[48], but recent data indicate that this may actually not be the case[26]. Our data indicate an increased intestinal absorption of cholate without changes in *Asbt* mRNA or Asbt protein levels (Figure 4) or Ibabp protein levels (data not shown) in STZ-diabetes. However, in diabetes several changes in intestinal morphology are evident. We found an increased weight of the ileum in STZ rats compared to controls (Figure 4). It has been reported that intestinal growth is enhanced in diabetes: the intestine becomes longer, has a larger diameter and a larger surface area for absorption. Finally, an increased mucosal width has been observed[49,50,51], and there appears to be an increased number of epithelial cells on intestinal villi and crypts[52]. In our experiment we collected the last 45 cm of the intestine and considered this to represent the ileum. However, as the intestine is longer in diabetes, we probably underestimated the total weight of the ileum in STZ-rats. Two in vitro studies examined the intestinal transport of bile salts in STZ diabetes. Thomson[53], using parts of rat intestine in incubation chambers and thereby excluding the enlargement of the intestine, found a greater uptake of taurocholic acid and a lower uptake of chenodeoxycholate and deoxycholate in the jejunum of diabetic rats compared to controls. In the ileum, the uptake of bile salts was similar compared to controls[53]. However, Caspary[54] reported that the rate of Na^+-dependent absorption of taurocholate and glycocholate into everted sacs of ileum was higher in diabetic than in control rats. Our data showed unchanged Asbt protein expression in STZ diabetes, but the total surface area for absorption was increased, associated with a 410% increase in daily cholate absorption. In insulin-treated STZ-rats cholate absorption was only increased by 160% compared to controls, while ileal weight was comparable to that in STZ-rats. The decreased

total amount of Asbt protein in STZ-ins rats therefore probably is responsible for the lower daily cholate absorption in these animals compared to STZ-rats.

In conclusion, the enlarged bile salt pool size in STZ-diabetic rats coincides with an increased synthesis of cholate and a concomitantly enhanced intestinal cholate absorption efficiency. Increased cholate synthesis appeared to be mainly attributable to enhanced 12-alpha-hydroxylation of bile salt intermediates due to increased hepatic expression of *Cyp8B1*. The enhanced cholate reabsorption in diabetes was not accompanied by adequate down-regulation of hepatic cholate synthesis. The inappropriately high rate of bile salt synthesis seemed independent of the diabetes-associated reduction of hepatic *Fxr* expression, because *Shp* mRNA levels were unaffected. In insulin-treated STZ-diabetic rats, bile salt pool size and cholate absorption were still increased compared to controls, creating an environment favouring lipid absorption and retention in the body. These factors could possibly contribute to the increased risk for atherosclerosis in diabetes mellitus type 1.

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7

General discussion

Type 1 diabetes mellitus and macrovascular disease

Macrovascular disease is the major cause of mortality in type 1 diabetes mellitus (DM1). After the age of 30 years the mortality rate due to coronary artery disease rises rapidly, equally in men and women[1,2,3]. For subjects with DM1 diagnosed in childhood, relative risks for CVD and total mortality are often 10-fold that of the general population[4].

Which factors in DM1 contribute to the early and profound development of atherosclerosis? As described in chapter 1, hyperglycemia may affect several processes leading to vasoconstriction, inflammation and a procoagulate state, and could thereby contribute to the development of atherosclerosis. As expected, improvement of metabolic control in DM1 was associated with a significant decrease in atherosclerosis in some studies[5,6]. However, in other studies and in a recent meta-analysis, no significant association could be demonstrated[7-10]. In the latter studies, atherosclerosis was associated with age, duration of diabetes, waist-to-hip ratio, blood pressure, BMI, smoking, albumin excretion rate, elevated triglyceride and decreased HDL cholesterol concentrations[7,8,9]. In the study by Orchard et al.[8], atherosclerosis in DM1 was associated with a decreased glucose disposal rate, indicating insulin resistance. Insulin resistance has been described as risk factor for CVD in DM1 by several authors[8,11,12]. Furthermore, various genetic polymorphisms associated with the development of complications in DM1 have been described[13-16]. All together, available data indicate that atherosclerosis in DM1 has a multifactorial etiology. The aim of the thesis was to unravel some factors involved in this process, focussing on lipid-, cholesterol- and bile salt metabolism.

Plasma lipids in type 1 diabetes mellitus

Increased triglycerides, VLDL- and LDL cholesterol and decreased HDL cholesterol has been associated with atherosclerosis in DM1[7-9,17-19]. According to the study of Renard et al. using diabetic mice, hyperglycemia accelerates arterial inflammation and atherosclerotic lesion initiation, while a diabetes-induced increase in VLDL will contribute to lesion progression and formation of advanced plaques[20]. In patients with DM1 in adequate glycemic control, plasma lipid levels are generally normal or even favorable[21,22]. **Chapter 2** and **3** address the potential disturbances in plasma lipid levels in children with type 1 diabetes. Fasting plasma triglyceride and cholesterol concentrations and non-fasting cholesterol, LDL cholesterol levels and cholesterol/HDL cholesterol ratio were positively correlated with HbA_{1c} percentages, in agreement with other studies in children and adolescents with DM1[23-26]. Female DM1 patients had higher cholesterol and LDL cholesterol concentrations than female controls and higher total cholesterol concentrations compared to male DM1 patients. In female children and adults with DM1, elevated total cholesterol and LDL cholesterol compared to male patients has been found[19,24,26,27]. Elevated

lipid levels in females with DM1 possibly contribute to the greater increase in coronary heart disease in women than men with DM1[1,3,28]. In contrast to other authors[23], LDL cholesterol and cholesterol/HDL cholesterol ratio was positively correlated with age. A less favorable metabolic control and slight insulin resistance with advancing age may have contributed to the rising lipid concentrations.

A delayed chylomicron (CM) clearance rate has been identified as risk factor for atherosclerosis in patients with coronary heart disease and in patients with type 2 diabetes[29,30]. In non-obese men with DM1 in poor control, a decreased CM clearance rate due to a decreased tissue uptake of CM remnants was described[31]. No studies on postprandial CM clearance in children or adolescents with DM1 have been reported so far. We investigated the clearance of CM in late teenagers with DM1 by measuring breath $^{13}CO_2$, plasma triglyceride, retinyl palmitate and ^{13}C-labeled oleic acid concentrations, after oral administration of a fat-rich meal containing vitamin A and ^{13}C-oleic acid (**chapter 2**). None of the three tests applied indicated a delay in CM clearance rate in late teenagers with DM1 compared with controls. The CM clearance rate was not related with metabolic control (HbA_{1c}), gender, fasting cholesterol, or triglyceride concentrations, indicating that the relatively higher triglyceride and cholesterol concentrations that were found in these teenagers with DM1, could not be attributed to a delayed chylomicron clearance. Possible explanations for the difference in outcome between our study and the study in adults with DM1[31] could be that the latter were in worse metabolic control compared to our patients. Another explanation for the difference between late teenagers and adults could be an age-dependency in CM clearance rate. In healthy adults, CM clearance rate, studied by vitamin A tolerance test, showed a progressive delay with increasing age[32]. CM clearance rate in DM1 could possibly be age-dependent, similar to the situation in individuals without DM1.

In conclusion, from our data, a delayed CM clearance rate at late teenager age appeared not to be a factor contributing to the increased risk for atherosclerosis in DM1. We did not study the composition of the lipoproteins. In adults with DM1 cholesterol enrichment of VLDL and LDL[33-36], and a preponderance of small dense LDL has been described[36,37]. In children with DM1 a predominance of small dense LDL, related to the degree of glycemic control has been found[25]. Other

Table 1: National Cholesterol Education Program classification of total and LDL-cholesterol levels in children and adolescents from families with hypercholesterolemia or premature cardiovascular disease[40]

Category	Total cholesterol mM (mg/dl)	LDL cholesterol mM (mg/dl)
Acceptable	< 4.4 (< 170)	< 2.8 (< 110)
Borderline	4.4-5.1 (170-199)	2.8-3.3 (110-129)
High	≥ 5.2 (≥ 200)	≥ 3.4 (≥ 130)

studies did not confirm these results and, in fact, revealed no difference or even larger LDL particles in children with DM1 compared to controls[38,39]. It is therefore unclear if changes in lipoprotein composition are already important in children with DM1. In adolescents with DM1 early signs of atherosclerosis have been found to be related to HDL and LDL cholesterol levels[24]. Regular control of lipid levels in children with type 1 diabetes is important, whereby lower cut-off values should be used than in adults, as serum cholesterol concentrations rise with age (see Table 1 for recommendations as proposed by the National Cholesterol Education Program[40] and Table 2 for recent recommendations from the American Diabetes Association[41,42].

Table 2: Target levels for lipid concentrations in children and adolescents with type 1 diabetes according to the American Diabetes Association[41,42].

Parameter	Target level
LDL cholesterol	< 2.6 mmol/l
HDL cholesterol	> 1.1 mmol/l
Triglycerides	< 1.7 mmol/l

Cholesterol metabolism in type 1 diabetes mellitus (figure 1)

In rats with streptozotocin-induced diabetes, increased cholesterol absorption and decreased hepatic cholesterol synthesis have been described[43]. In adults with type 1 diabetes, cholesterol synthesis also appeared decreased and cholesterol absorption increased compared to patients with type 2 diabetes and to non-diabetic controls[44,45,46]. An increase in cholesterol absorption has been associated with a decrease in hepatic LDL-receptor activity, thereby contributing to a delayed LDL clearance[47]. To the best of our knowledge, no data on cholesterol absorption and synthesis in children with DM1 have been published. In **chapter 3** cholesterol synthesis and absorption was investigated in children with DM1. In DM1 children, the cholesterol synthesis marker (plasma lathosterol/cholesterol ratio) was significantly lower compared to controls, in agreement with recent data in adults with DM1[44,45,46]. Indices for cholesterol absorption were increased in absolute concentrations in DM1 patients, but were comparable with controls when expressed relative to cholesterol concentrations. The reason for not finding an increase in cholesterol absorption markers in DM1, in contrast to studies in adults[44,45,46], may be caused by differences in study method. In contrast to the studies mentioned[44,45,46], samples were obtained under non-fasting conditions and subjects had not used a controlled diet. On the other hand, it could be that, at this age, cholesterol absorption is still unaffected. Other factors may have influenced cholesterol concentration in children with DM1, for example a decreased LDL receptor expression[18,48]. In DM1 patients and controls, cholesterol absorption

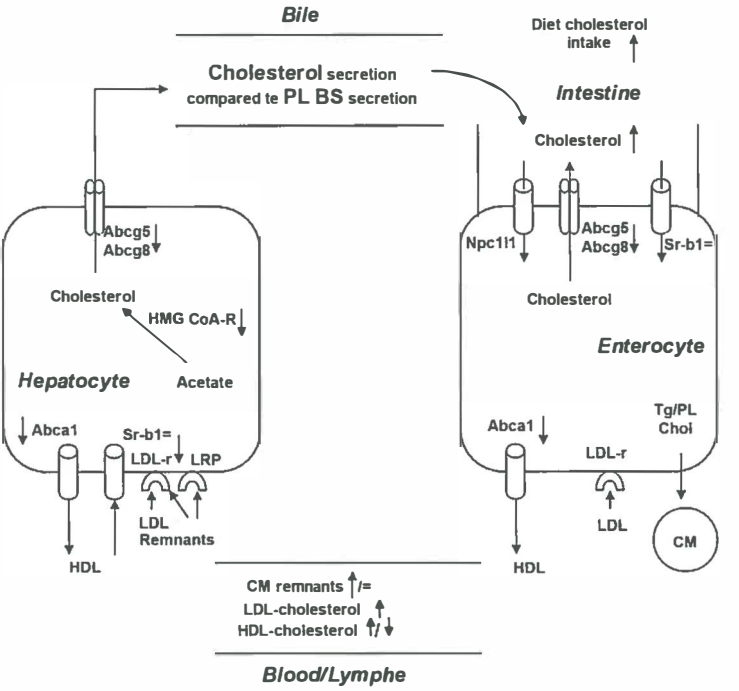

Figure 1. Schematic overview of cholesterol metabolism in type 1 diabetes mellitus. Hyperphagia in STZ-diabetic rats will lead to an increased dietary cholesterol intake compared to control rats. A decreased expression of the ATP binding cassette transporters G5 and G8 (Abcg5, Abcg8) may lower the transport of cholesterol from the enterocyte back into the intestinal lumen, thereby contributing to elevated cholesterol absorption. A decreased expression of the ATP binding cassette transporter A1 (AbcA1) on enterocytes and hepatocytes may contribute to a lowered HDL cholesterol concentration. A decreased LDL-receptor (LDL-r) expression and a decreased heparan sulfate proteoglycan production (see **chapter 1**) may lead to elevated LDL cholesterol and chylomicron remnants concentrations. Hepatic cholesterol synthesis in DM1 is decreased and associated with lowered hydroxy-methylglutaryl coenzyme A reductase (HMG CoA-R) expression. Biliary cholesterol secretion is decreased relative to phospholipid (PL) and bile salt (BS) secretion, probably due to decreased expression of hepatic Abcg5/Abcg8 expression.

decreased with age, whereas cholesterol synthesis increased with age. Age was positively correlated with daily insulin dosage indicative for deteriorating insulin sensitivity during puberty. Mild insulin resistance of puberty has been described in children without diabetes[49,50] and could play a role in the control group. Insulin resistance has been associated with increased cholesterol synthesis and decreased cholesterol absorption[51]. Therefore, the mild insulin resistance of puberty could contribute to the rising lathosterol- and decreasing plant sterol ratios with advancing age observed in patients and controls.

To study underlying factors for the observed changes in cholesterol metabolism, we investigated intestinal cholesterol absorption and biliary cholesterol excretion

in rats with streptozotocin (STZ)-induced diabetes (See **chapter 4**). In STZ-diabetic rats, biliary cholesterol excretion, relative to secretion of bile salts, or phospholipids, was decreased compared with controls. Cholesterol absorption, measured by plasma plant sterol concentrations and calculated by subtracting the daily faecal neutral sterol loss from daily biliary cholesterol excretion rates plus estimated daily dietary cholesterol intake, was increased in diabetic animals compared to controls. Cholesterol absorption and hepatobiliary cholesterol excretion partially normalized upon insulin supplementation.

Increased cholesterol absorption in the diabetic state has been associated with hyperphagia[43,52], an enlarged bile salt pool size, creating an environment favoring cholesterol absorption (**chapter 6**, [53,54]), and an enlarged intestinal absorptive area (**chapter 6**, [46,55-57]). Recent studies have shown that the ATP binding cassette halftransporters Abcg5 and Abcg8 are involved in the transport of cholesterol from the enterocyte back into the intestinal lumen[58,59,60]. In streptozotocin-induced diabetic rats, the intestinal mRNA expression of *Abcg5* (-47%) and *Abcg8* (-43%) as well as Abcg5 protein contents were reduced compared to controls, thereby probably lowering the efflux of cholesterol from the enterocyte to the intestine. Reduced Abcg5 and Abcg8 expression in the diabetic state could therefore contribute to the increased cholesterol absorption in diabetes. Recently, the transporter Niemann-Pick C1 Like 1 (NPC1L1) has been shown to be critical for the uptake of cholesterol from the intestine into the enterocyte[61,62]. Further research is necessary to evaluate if an increased expression of NPC1L1 contributes to the enhanced cholesterol absorption in diabetes.

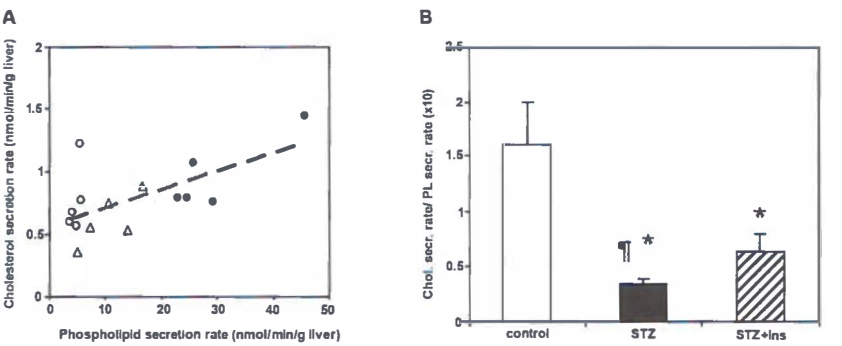

Figure 2. Changes in biliary cholesterol- and phospholipid secretion rate upon induction of streptotocin-diabetes in rats. **A:** Biliary cholesterol secretion rate (nmol/min/g liver) compared to phospholipid secretion rate (nmol/min/g liver) in controls (open circles), STZ-diabetic rats (closed circles) and insulin treated STZ-rats (open triangles). Cholesterol secretion rate was correlated with phospholipid secretion rate with the following equation: [Cholesterol secretion rate] = 0.0144[phospholipid secretion rate] + 0.5468 ($r = 0.65$, $p = 0.01$). **B:** Biliary cholesterol secretion rate expressed relative to phospholipid secretion rate. *Open bars*, control rats; *closed* bars, diabetic rats; *striped bars*: diabetic rats treated with insulin. Mean values ± SD of five rats per group, asterisks indicates significant difference from control values; ¶ indicates significant from insulin-treated STZ-rats.

Under normal conditions, biliary cholesterol secretion is coupled to phospholipid secretion in a process that is, in part, controlled by bile salt secretion. In streptozotocin-induced diabetic rats biliary phospholipid and bile salt secretion is increased compared to controls (as described in **chapter 6**). Accordingly one would expect that biliary cholesterol secretion would be enhanced in diabetic rats. As is illustrated in Figure 2, biliary cholesterol secretion rate is associated with biliary phospholipid secretion rate, however, STZ-diabetic and insulin-treated STZ-diabetic rats secreted much less cholesterol relative to phospholipids than control rats. In patients with type 1 diabetes biliary cholesterol excretion was lower compared to controls[63]. As described in **chapter 4**, a defective HDL cholesterol uptake appeared not to be contributing to the decreased biliary cholesterol excretion rate in the diabetic rats. Theoretically, a decrease in cholesterol synthesis could influence the biliary cholesterol secretion. In STZ-diabetic rats, *HMG CoA reductase* mRNA levels were decreased compared to controls, yet, the increased cholesterol absorption will lead to an increased hepatic cholesterol influx. Hepatic free cholesterol content was similar in rats with and without STZ-diabetes. In STZ-diabetic rats the hepatic expression of the ABC-transporters *Abcg5* and *Abcg8* mRNA levels were strongly reduced compared with control animals. Therefore, reduced Abcg5 and Abcg8 expression could contribute to the decreased hepatic cholesterol secretion in the diabetic state.

Which factor could cause *Abcg5/Abcg8* down-regulation in liver and intestine in STZ-diabetic rats? The transcription of both genes is stimulated by Liver X receptor (LXR). Insulin has been shown to stimulate *Lxr* mRNA levels in cultured hepatocytes and in livers of rats and mice injected with insulin[64]. In the STZ-rats, decreased insulin concentrations might lead to lower LXR expression, however, at mRNA level *Lxr* expression was not significantly different from controls. Furthermore, metabolic consequences of DM1 (high concentrations of free fatty acids and ketone bodies) may antagonize activation of LXR by oxysterols, thereby inhibiting transcription of LXR target genes[65,66,67].

In the insulin treated diabetic rats, biliary cholesterol secretion and hepatic *Abcg5* and *Abcg8* expression were still decreased compared with controls. Also cholesterol absorption in these rats, as measured by plant sterol concentrations was increased and intestinal Abcg5 protein expression was still decreased compared with controls. In adults, but not in children with type 1 diabetes, cholesterol absorption was significantly elevated compared to controls. If altered expression of Abcg5 / Abcg8 transporters may also be associated with the increased cholesterol absorption in DM1 patients has not yet been investigated.

Cholesterol synthesis was decreased in children and adults with DM1 compared with controls and patients with type 2 diabetes[44,45,68]. In children and adults with DM1, markers of cholesterol synthesis were strongly correlated with markers of cholesterol absorption[44,45,68]. Enhanced cholesterol absorption has been associated

with a decreased LDL-receptor activity and decreased cholesterol synthesis[47]. Insulin administration in mice induced the expression of genes involved in cholesterol biosynthesis (HMG-CoA reductase, squalene synthase), which in part was associated with LXR[64]. Therefore insulin deficiency could in part be associated with decreased cholesterol synthesis due to lowered LXR signaling.

Intervention studies in patients with type 1 diabetes and STZ-diabetic rats, in which cholesterol absorption is inhibited, e.g., by plant stanol esters (margarine), and the effects hereof on markers of cholesterol absorption, cholesterol synthesis and plasma cholesterol levels and Abcg5/Abcg8 expression still have to be performed. Few studies with plantsterol/stanol esters-enriched spreads in type 2 diabetes have been performed and show modest cholesterol lowering effects, that still may be beneficial in terms of cardiovascular disease risk reduction[69,70].

Bile salt metabolism in type 1 diabetes mellitus (figure 3)

In diabetes several changes in bile salt metabolism have been described. In humans few studies have been performed and showed an increased bile salt pool size and bile salt synthesis in patients with type 2 diabetes[71,72]. In experimental type 1 diabetes, the composition of bile appeared to be altered. The biliary secretion rate of bile salts and phospholipids is profoundly increased[53,73,74], whereas biliary glutathione secretion has been shown to be decreased[75]. The secretory rate maximum (SRm) of bile salts, i.e., the maximal amount of a certain bile salt that can be secreted into bile per unit of time before cholestasis is induced, appeared to be higher in alloxan-induced diabetic rats than in controls[73]. The mechanisms responsible for these changes in bile formation and composition in experimentally-induced diabetes are still largely unknown. In **chapter 5** changes in the hepatic expression of various ABC transporters in the hepatic canalicular membrane were associated with changes in bile formation in the diabetic state. A key finding was the increased expression of Abcb4 (multidrug resistance P-glycoprotein type 2 or Mdr2-Pgp) at mRNA (+105%) and protein (+530%) level. The increased Abcb4 expression was associated with a 520% increase in biliary phospholipid secretion. Insulin treatment lowered biliary phospholipid secretion and Abcb4 levels. Because phospholipid secretion is stimulated by bile salt secretion[76], the increased bile salt output in the diabetic state could contribute to these findings. However, biliary phospholipids to bile salt ratios were increased in STZ-diabetic rats (+77% under basal conditions, +176% during maximal bile salt stimulation), indicating an effect independent of bile salt excretion. In rats, hepatic *Abcb4* expression was induced by cholate feeding[77]. Therefore, the increased cholate pool size in STZ-diabetes (Chapter 6) may induce *Abcb4* expression. The nuclear receptor PPARα mediates induction of Abcb4 gene expression[78,79]. In diabetes, the increased flux of free fatty acids to the liver could induce *Abcb4* expression via PPARα. Abcb4 gene expression is also activated by the nuclear receptor FXR. However, as is

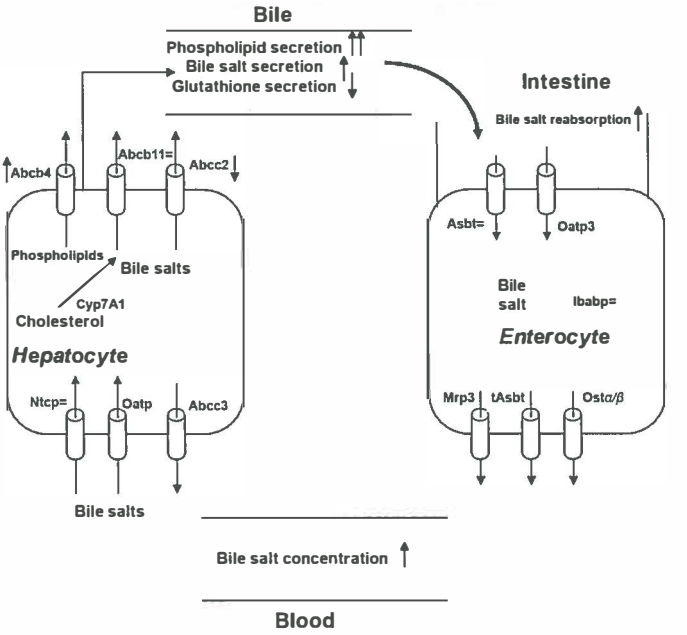

Figure 3. Schematic overview of bile salt metabolism in type 1 diabetes mellitus.
Biliary bile salt secretion is increased in STZ-diabetic rats and associated with a strongly increased biliary phospholipid secretion. The increased hepatic expression of Abcb4 (multidrug resistance P-glycoprotein type 2 or Mdr2) has been associated with the increased biliary phospholipid secretion. Abcb11 (bile salt export pump or Bsep) expression is unchanged despite the increased biliary bile salt excretion (see chapter 5). The decreased expression of Abcc2 (multidrug resistance-associated protein 2 or Mrp2) has been associated with the decreased biliary gluathione secretion and the lowered bile salt-independent bile flow found in the diabetic state. Bile salt synthesis is increased in diabetes, however, with no changes in cholesterol 7o-hydroxylase mRNA (Cyp7A1). The expression of the Na^+-taurocholate co-transporting polypeptide (Ntcp) involved in hepatic uptake of bile salts was unchanged in diabetes. Protein concentrations of the apical sodium- dependent bile salt transporter (Asbt) and the ileal bile acid-binding protein (Ibabp) were comparable between rats with and without diabetes. However, bile salt reabsorption is increased in DM1 possibly due to intestinal modifications (see Chapter 6).

shown in chapter 6 and by Duran-Sandoval, et al.[80], FXR expression is decreased in STZ-diabetes. Therefore, FXR is most likely not involved in inducing *Abcb4* gene expression under diabetic conditions.

In rats with STZ-diabetes, the expression of the major canalicular transporter for bile salts Abcb11 (bile salt export pump or Bsep), was slightly increased at mRNA level (+60%), but unchanged at protein levels. Yet, biliary bile salt output was increased by 260% in STZ-rats compared to controls. *Abcb11* gene expression is positively regulated by FXR[81,82]. As described previously, hepatic FXR expression is decreased in STZ-diabetes and thereby seems not important in *Abcb11* gene upregulation in the diabetic state. The strongly increased biliary bile salt secretion

in STZ-diabetic rats with unchanged Abcb11 protein expression implicates a considerable overcapacity of this transport system. This was also shown by the measurement of the secretory rate maximum (SRm). As described in chapter 5, the SRm, for sodium taurocholate appeared to be increased by 130% in STZ-rats compared to controls, whereas SRm values in insulin-treated diabetic rats were intermediate. We hypothesize that the strongly enhanced phospholipid secretion, governed by the increased Abcb4 expression, shields the hepatic canalicular membrane against the detergent effect of the secreted bile salts. This facilitates the increased SRm for taurocholate in the STZ-diabetic state. Confirmation of this hypothesis could possibly be obtained by inducing diabetes in *Abcb4* knockout mice.

Hepatic protein levels of Abcc2 (multidrug resistance-associated protein 2 or Mrp2), the transporter involved in the biliary secretion of glutathione, appeared markedly reduced in STZ-diabetic rats and remained depressed after insulin treatment. Abcc2 protein expression is most likely regulated at a posttranscriptional level, because no changes were detected in *Abcc2* mRNA. The decreased Abcc2 protein expression is most likely responsible for the reduction of the bile salt-independent bile formation that has been reported in STZ-diabetic rats[75], as well as in spontaneously diabetic biobreeding rats[83]. Biliary glutathione has been identified as the major driving factor in the generation of the bile salt-independent bile flow in rodents[84]. Insulin treatment had only a slight effect in restoring Abcc2 expression and biliary glutathione concentrations. If the bile salt-independent bile flow is also decreased in patients with diabetes, this could possibly interfere with the bile dependent excretion of certain drugs[85].

In **chapter 6** the kinetics of bile salt metabolism were determined by isotope dilution using $^{2}H_{4}$-cholate in rats with streptozotocin (STZ)-induced diabetes, control rats and insulin-treated STZ-rats (STZ-ins rats). In the diabetic state, cholate pool size and cholate synthesis rate were increased by 535% and 106%, respectively. The estimated cholate absorption per enterohepatic cycle (as percentage of the cholate pool size) increased from 96.2% in controls to 98.2% in STZ rats. The calculated amount of cholate that was absorbed per day increased by 410% in STZ rats. These data imply that the enlarged cholate pool size in diabetes is associated with both an increased synthesis rate and more efficient cholate absorption. Therefore, the "normally" occurring feedback mechanism in which enhanced bile salt absorption will induce a decrease in bile salt synthesis appeared abolished in the diabetic state. In insulin-treated diabetic rats, cholate kinetic data shifted towards control values, but cholate pool size and cholate absorption were still significantly increased compared to control rats.

The reason why the increased cholate absorption in STZ-diabetes did not induce repression of bile salt synthesis is unknown. The bile salt feedback mechanism is (in part) controlled by the farnesoid X receptor (FXR)[86,87,88]. In STZ-diabetes we

and others found a reduced mRNA expression of *Fxr*, which has been attributed to prevailing low intracellular glucose concentrations[80], however, *Shp* expression was comparable compared to controls. Due to the indirect effect of FXR via SHP, it seems unlikely that FXR-SHP mediated the ineffective feedback response on bile salt synthesis. Further research in FXR knockout mice with STZ-induced diabetes or in rats treated with an FXR-agonist could possibly unravel the effect of the decreased FXR in STZ diabetes. However, as *Cyp7A1* mRNA levels were unchanged in STZ-diabetes, the increase that was found in total bile salt synthesis (+50%) is most likely attributable to Cyp7A1 activity regulation at a post-transcriptional level. On the other hand, it should be realised that livers were harvested during daytime. Since *Cyp7A1* expression shows a marked day-night variation, it cannot be excluded that expression was higher during the night in diabetics.

The 106% increased cholate synthesis in STZ diabetes was associated with a strongly increased Cyp8B1 expression. The increased Cyp8B1 expression probably contributed to the increased biliary and faecal cholate / chenodeoxycholate derivates ratio in STZ diabetes. As described in chapter 6 we speculate that the increased *Cyp8B1* transcription may be attributed to increased PPARα activity due to increased fatty acid fluxes in the diabetic state. Further studies in PPARα-knockout mice with STZ-induced diabetes could generate more information on this topic.

In STZ-diabetes, the elevated intestinal cholate absorption was not associated with changes in the expression of the apical sodium-dependent bile salt transporter (Asbt). We speculate that the changed intestinal morphology in the diabetic state with an increased absorptive area and an increased mucosal width may have caused underestimation of the number of Asbt transporters. Measuring the exact length and mucosal width of the intestinal part used for Asbt protein measurement could possibly confirm this hypothesis. Further information could be given by diabetes induction in Asbt-knockout mouse models or by admitting Asbt inhibiting drugs to STZ-diabetic rats.

Whether the above-mentioned data on bile salt metabolism in STZ-diabetes have consequences for patients with type 1 diabetes is unclear. The insulin-treated STZ-rats showed still an elevation of cholate pool size and cholate absorption and a trend to increased cholate synthesis ($P>0.05$). Few studies have been performed in patients with diabetes with variable results. In patients with poorly controlled type 2 diabetes, an increased bile salt pool size and bile salt synthesis without changes in biliary bile salt composition has been described[71]. In patients with type 1 diabetes, a decreased biliary excretion of bile salts was described with an increase in secondary bile salts and an increased glycine/taurine ratio[63]. The lower biliary bile salt concentration in DM1 was attributed to a more sluggish gallbladder. The changes in biliary bile salt composition were attributed to a decreased motility of the distal small intestine, or a decreased absorption of bile

salts in the terminal ileum with enhanced bacterial modification of bile salts[63]. In contrast, Andersén et al. found no changes in biliary bile salt composition in patients with type 1 diabetes compared to controls, but an increased percentage of deoxycholate and a reduced percentage of cholate in bile of patients with type 2 diabetes[89]. Studies in rats can give different results compared to humans, because in rats, in contrast to humans, absorbed deoxycholate can be converted to cholate and add to the cholate pool. To investigate this effect, a dual label test with labeled cholate and deoxycholate at different sites can possibly be used. Furthermore in humans and in mice ASBT expression is inhibited by bile salts via FXR/SHP/LRH1, while in rats this feedback mechanism does not occur[90,91]. Decreased ASBT expression could possibly contribute to the relatively increased biliary deoxycholate concentrations described in diabetes and to diarrhoea due to bile salt malabsorption in diabetes[92].

Concluding remarks

The above-described research tried to unravel some aspects of lipid-, bile salt- and cholesterol metabolism in type 1 diabetes. From recent literature, it appears that not only the absence of insulin, but also the metabolic changes caused by insulin deficiency (elevated free fatty acid and acetoacetate levels, decreased intracellular glucose levels, increased AGEs) induce alterations in transcription factors and transporter proteins associated with cholesterol and bile salt metabolism. Further research in knockout mouse models or cultured hepatocytes kept under controlled metabolic conditions may give more information on the factors inducing the alterations in expression of transcription factors and transport proteins.

What implications could the described results have for patients and especially children with type 1 diabetes? Some changes in lipid metabolism in type 1 diabetes (delayed chylomicron clearance) are not yet evident in this age group. However, early signs of atherosclerosis were described in children and adolescents with type 1 diabetes[24,93-96]. Krantz et al. showed that increased intima-media thickness in adolescents with type 1 diabetes was associated with diabetic complications (hypertension, retinopathy, nephropathy) and lipoprotein levels, but not with HbA_{1c}. However, HbA_{1c} was significantly correlated with lipid levels, suggesting that glycemic control could have an indirect effect on atherosclerosis through lipids[24]. LDL cholesterol and HbA1c were positively correlated with aortic and coronary atherosclerosis in autopsy studies[97,98,99]. These studies demonstrated that aortic and coronary atherosclerosis are commonly seen before the age of 20 years. In our studies, described in **chapter 2** and **3,** HbA_{1c} was positively correlated with cholesterol, LDL cholesterol, cholesterol/HDL cholesterol ratio and fasting triglycerides, as has been described in other studies in children and adolescents with DM1[23-26]. Therefore optimization of glucose control in DM1 should initially be the major goal of therapy.

According to recent guidelines from the American Diabetes Association, treatment of dyslipidemia in children and adolescents with DM1 should be guided by fasting lipid levels, (mainly LDL) obtained after glucose control is established[41,42]. Initial therapy should consist of optimization of glucose control and medical nutrition therapy aimed at a decrease in the amount of total and saturated fat in the diet, as well as encouragement of life style changes to control weight, increase exercise, and if applicable discontinue tobacco use. The addition of pharmacologic lipid lowering agents is recommended for LDL > 4.1 mmol/l. Medication is also recommended in patients who have LDL cholesterol values of 3.4 – 4.1 mmol/l in the presence of a high cardiovascular risk factor. Treatment should aim to lower LDL levels to < 2.6 mmol/l. Bile acid sequestrants (cholestyramine, colestipol) are generally recommended as first choice treatments in this age group[40]. However, they are not well tolerated and compliance is poor. Furthermore, use of bile salt sequestrants is known to elevate plasma triglyceride levels. Therefore, statins (HMG-CoA reductase inhibitors) are currently also advised for use in children. Short term trials with statins in children and adolescents with familial hypercholesterolemia have confirmed their safety and efficacy[100,101,102]. Few short-term clinical trials with statins have been performed in adults with DM1[103,104,105]. In these studies LDL cholesterol concentrations were significantly lowered compared with untreated DM1 patients. Yet, no differences in micro- or macrovascular complications were found, possibly due to the short time period of the studies. Pravastatin treatment was associated with CPK elevation[104]. The Heart Protection Study using statins for 5 years in 5963 adults with diabetes, 10% of whom had type 1 diabetes, showed a reduced risk of first myocardial infarction, stroke and limb-revascularisation by 25%. The risk reduction in type 1 patients was similar to that in type 2 patients, but was statistically insignificant[106]. In children with DM1, cholesterol synthesis appeared decreased compared with controls (**chapter 3**), which might be associated with increased cholesterol absorption. Pharmacological agents inhibiting cholesterol absorption such as ezetimibe could therefore be a more effective drug for lowering plasma cholesterol concentrations. Cholesterol absorption could be inhibited at first by plantsterol/stanol enriched food and later on be combined with ezetimibe. A decrease in cholesterol absorption will reduce the flux of cholesterol to the liver. The decreased cholesterol flux to the liver will lead to an increased LDL receptor expression, thereby lowering plasma LDL cholesterol concentrations. However, the liver will compensate for the loss of cholesterol returning to the liver from the intestine by up regulating cholesterol biosynthesis, which could mitigate the LDL lowering effect. If the LDL cholesterol goal is not achieved a statin could be added to the treatment protocol.

As was shown in **chapter 4**, reduced intestinal and hepatic Abcg5 and Abcg8 transporter expression could contribute to the increased intestinal cholesterol absorption and decreased biliary cholesterol excretion in the diabetic state and was

still evident in insulin treated diabetic rats. Can the expression of these transporters be stimulated? In sitosterolemia, a disease resulting from mutations in ABCG5 or G8 transporters, ezetimibe is successfully used to lower plasma plantsterol and cholesterol concentrations[107]. Ezetimibe interferes with the Niemann-Pick C1-Like 1 (NPC1L1) transporter that is most likely responsible for uptake of cholesterol and plant sterols by the intestine[108]. The transcription of *Abcg5/Abcg8* is stimulated by the Liver X receptor (LXR)[109]. Synthetic LXR agonists have been generated and increase hepatobiliary cholesterol excretion and reduce intestinal cholesterol absorption via Abcg5/g8[110]. However, as LXR also stimulates *Srebp-1c* leading to the formation of fatty acids and triglycerides, hepatic steatosis and elevated plasma triglycerides are serious side effects of synthetic LXR agonists[111]. Kaneko et al. developed LXR agonists from phytosterols that selectively induced intestinal *Abcg5* and *Abcg8* expression thereby reducing intestinal cholesterol absorption, without inducing hypertriglyceridemia[112]. Possibly these selective LXR agonists could be indicated in the treatment of dyslipidemia in type 1 diabetes.

In **chapter 5** and **6,** we showed that biliary bile salt secretion, bile salt pool size, bile salt synthesis and intestinal bile salt reabsorption were increased in STZ-diabetic rats compared with controls. It still has to be elucidated, if and to what extent, bile salt metabolism is changed in humans with DM1. An increased bile salt synthesis would be favourable as a way to excrete cholesterol from the body. However, the increased bile salt secretion in the intestine and the increased bile salt reabsorption would unfavourably lead to higher cholesterol absorption. In that case, bile acid sequestrants could be worthwhile in decreasing plasma LDL cholesterol levels. Binding of bile salts in the intestine will reduce the enterohepatic circulation of bile salts, thereby stimulating the hepatic conversion of cholesterol into bile salts. The decreased hepatic cholesterol content will enhance LDL receptor expression, leading to lower plasma LDL cholesterol concentrations. Gastrointestinal side effects and form of administration (powder) lead to poor compliance. Newer bile acid sequestrants in tablet form and with fewer side effects may improve adherence.

To establish the long-term efficacy and safety of lipid lowering drugs in children and adolescents with type 1 diabetes for lifelong primary prevention of CVD, randomised clinical trials are needed. Furthermore, the optimal age for initiating pharmacological intervention in type 1 diabetes still has to be defined.

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SUMMARY

Type 1 diabetes mellitus (DM1) is associated with microvascular complications (retinopathy, nephropathy, neuropathy) and macrovascular complications (cardiovascular disease). Macrovascular disease is the major cause of death in patients with DM1. The mortality rate due to coronary artery disease in DM1 rises rapidly after the age of 30 years. Atherosclerosis in DM1 has been associated with compromised metabolic control (hyperglycemia, HbA_{1c}), yet, in several studies and in a recent meta-analysis no correlation with HbA_{1c} was found. Other factors that have been associated with atherosclerosis in DM1 are smoking, hypertension, insulin resistance, nephropathy, specific genetic factors and disturbances in plasma lipid concentrations. All together, available data indicate that the enhanced atherosclerosis in DM1 has a multifactorial etiology. The aim of the thesis was to determine the contribution of specific factors in this process, focussing on lipid-, cholesterol- and bile salt metabolism. Thus, factors related to postprandial lipid- and cholesterol metabolism were investigated in children and adolescents with DM1, since it is anticipated that derangements of metabolism and development of atherosclerosis start already at a young age. Furthermore, molecular mechanisms underlying disturbed lipid-, cholesterol- and bile salt metabolism were evaluated in an established animal model of DM1.

Chapter 1 provides a general overview of pathophysiologic features of macrovascular disease and lipoprotein metabolism and the consequences of DM1 hereupon. Available studies on atherosclerosis and lipid levels performed in children with DM1 are summarized. We describe the cholesterol and bile salt metabolism, thereby addressing relevant receptors, transport proteins and transcription factors.

A decreased clearance rate of chylomicrons (CM) has been associated with atherosclerosis in adults with DM1. No information on CM clearance rate in children and adolescents with DM1 is available. In **chapter 2** we investigated the CM clearance rate in teenagers with DM1 by measuring breath $^{13}CO_2$, plasma triglyceride, retinyl palmitate and ^{13}C-oleic acid concentrations after oral administration of a fat-rich meal containing vitamin A and ^{13}C-oleic acid. None of the tests applied indicated a delay in CM clearance rate in teenagers with DM1 compared to controls. In DM1 patients, fasting triglyceride and cholesterol concentrations were positively correlated with the level of metabolic control, as characterized by HbA_{1c}. CM clearance rate was not related with either HbA_{1c}, gender, fasting cholesterol or triglyceride concentrations in teenagers with DM1. The difference in outcome between children and adults with DM1 could possibly be explained by a worse metabolic control in adult patients, or due to an age-dependency in CM clearance rate. In healthy adults, CM clearance rate showed a progressive delay with increasing age.

LDL cholesterol in DM1 can be elevated by a variety of factors. In rats with streptozotocin (STZ)-induced diabetes (STZ-diabetes is used as a model for DM1) intestinal cholesterol absorption was increased compared with control rats. This increased cholesterol absorption was associated with a decreased hepatic cholesterol synthesis and suppressed hepatic LDL receptor expression. These data suggest that hyperabsorption of (dietary) cholesterol in DM1 leads to a delayed LDL clearance. In adults with DM1, markers of cholesterol absorption were increased and markers of cholesterol synthesis decreased compared with controls and adults with type 2 diabetes. So far, no studies have been reported on cholesterol absorption and synthesis in children and adolescents with DM1. In **chapter 3** cholesterol synthesis and absorption was investigated in children with DM1. In DM1 children, a cholesterol synthesis marker (plasma lathosterol/ cholesterol ratio) was significantly lower compared with controls, in agreement with recent data in adults with DM1. Absolute values of markers of cholesterol absorption (cholestanol, campesterol and sitosterol) were significantly higher in DM1, yet, were comparable after correction for plasma cholesterol concentration. Possibly, the non-fasting conditions under which blood samples were taken and not using a controlled diet in our study, in contrast to the studies in adults with DM1, may have influenced the outcome. Advancing age in DM1 patients and controls was associated with increasing markers of cholesterol synthesis and decreasing cholesterol absorption markers. Puberty has been associated with a decreased insulin sensitivity, as was shown in children with DM1 by a positive correlation between age and daily insulin dosage (per kg body weight). In adults, insulin resistance has been associated with increased cholesterol synthesis and decreased cholesterol absorption. Therefore, relatively mild insulin resistance may have contributed to the age-related changes in cholesterol synthesis and absorption.

To study underlying factors for the observed changes in cholesterol metabolism, we investigated intestinal cholesterol absorption and biliary cholesterol excretion in rats with STZ-induced diabetes **(chapter 4)**. In STZ-diabetic rats, biliary secretion of cholesterol, relative to the secretion of either bile salts or phospholipids, was decreased compared with controls. Cholesterol absorption, measured by plasma plant sterol concentrations and calculated by subtracting the daily faecal neutral sterol loss from daily biliary cholesterol secretion rates plus estimated daily dietary cholesterol intake, was increased in diabetic animals compared with controls. Cholesterol absorption and hepatobiliary cholesterol secretion partially normalized upon insulin supplementation.

Recent studies have shown that the ATP binding cassette halftransporters Abcg5 and Abcg8 are involved in biliary cholesterol secretion and in the transport of cholesterol from the enterocyte back into the intestinal lumen. In STZ-diabetic rats, the relatively decreased biliary cholesterol secretion was associated with

a reduced hepatic expression of *Abcg5* and *Abcg8*. Accordingly, the increased intestinal cholesterol absorption was associated with reduced intestinal mRNA and protein expression of *Abcg5* and *Abcg8*. This indicates that the relatively decreased biliary cholesterol secretion and increased cholesterol absorption may in part be attributed to a decreased hepatic and intestinal *Abcg5* and *Abcg8* expression. Transcription of both *Abcg5* and *Abcg8* is controlled by the Liver X receptor (LXR). LXR expression is stimulated by insulin. In the STZ-rats, decreased insulin concentrations might lead to lower LXR expression and thereby decreased *Abcg5/g8* expression. However, at mRNA level, hepatic and intestinal *Lxr* expression were not significantly different from controls. Free fatty acids and acetoacetate are increased in the diabetic state and may antagonize the activation of LXR by oxysterols. When LXR is less activated due to effects of FFA's or acetoacetate, transcription of *Abcg5/g8* will be decreased. In conclusion, the decreased biliary cholesterol secretion and the increased intestinal cholesterol absorption in diabetes is associated with decreased hepatic and intestinal Abcg5 and Abcg8 expression, probably due to metabolic interference with LXR activity.

In diabetes several changes in bile salt metabolism have been described. Patients with type 2 diabetes showed an increased bile salt pool size and bile salt synthesis. In animal models of DM1, a profound increase in biliary bile salts and phospholipids secretion and a decrease in biliary glutathione secretion has been found. The secretory rate maximum (SRm) of bile salts, i.e., the maximal amount of a certain bile salt that can be secreted into bile per unit of time before cholestasis is induced, appeared to be higher in diabetic rats than in controls. The mechanisms responsible for these changes in bile formation and composition in diabetes are still largely unknown. In **chapter 5** changes in the hepatic expression of various ABC transporters in the hepatic canalicular membrane were associated with changes in bile formation in the diabetic state. A key finding was the increased expression of the phospholipid transporter Abcb4 (the multidrug resistance P-glycoprotein type 2, Mdr2-Pgp) at mRNA (+105%) and protein (+530%) level. The increased Abcb4 expression was associated with a 520% increase in biliary phospholipid secretion. Insulin treatment lowered biliary phospholipid secretion and Abcb4 levels. Surprisingly, in rats with STZ-diabetes, the expression of the major canalicular transporter for bile salts Abcb11 (bile salt export pump, Bsep) was slightly increased at mRNA level (+60%), but unchanged at protein levels, whereas biliary bile salt output was increased by 260%. In addition, the secretory rate maximum (SRm) for sodium taurocholate appeared to be increased by 130% in STZ-rats compared with controls, whereas SRm values in insulin-treated diabetic rats were intermediate. The strongly increased biliary bile salt secretion in STZ-diabetic rats with unchanged Abcb11 protein expression implicates a considerable overcapacity of this transport system. We hypothesize that the strongly enhanced phospholipid secretion, governed by the increased Abcb4 expression, shields the

hepatic canalicular membrane against the detergent effect of the secreted bile salts. This facilitates the increased SRm for taurocholate in the STZ-diabetic state. Hepatic protein levels of Abcc2 (multidrug resistance-associated protein 2, Mrp2), the transporter involved in the biliary secretion of glutathione, appeared markedly reduced in STZ-diabetic rats. Insulin treatment had only a slight restoring effect on Abcc2 expression and biliary glutathione secretion. Biliary glutathione is important for the generation of the bile salt-independent bile flow. In STZ-diabetic rats, as well as in spontaneously diabetic biobreeding rats, a reduction of the bile salt-independent bile formation has been reported and this is probably caused by the lowered Abcc2 expression and a consequently decreased glutathione secretion. When a lowered bile salt-independent bile flow is also present in patients with DM1, it could possibly interfere with the biliary excretion of certain drugs.

In **chapter 6** the kinetics of bile salt metabolism were determined by isotope dilution using $^{2}H_{4}$-cholate in rats with streptozotocin (STZ)-induced diabetes, control rats and insulin-treated STZ-rats. In the diabetic state, cholate pool size and cholate synthesis rate were increased by 535% and 106%, respectively. Besides, the calculated amount of cholate that was absorbed per day increased by 410% in diabetes. These data imply that the enlarged cholate pool size in diabetes is associated with both an increased synthesis rate and more efficient cholate absorption. Therefore, the physiological feedback mechanism in which enhanced bile salt absorption will induce a decrease in bile salt synthesis appeared abolished in the diabetic state. Insulin-treatment only partly normalized the cholate kinetic data.

The reason why the increased cholate absorption in STZ-diabetes did not induce repression of bile salt synthesis is unknown. The bile salt feedback mechanism is (in part) controlled by the farnesoid X receptor (FXR). In STZ-diabetes, we and others found a reduced mRNA expression of *Fxr*, which has been attributed to prevailing low intracellular glucose concentrations. However, the expression of the Fxr target gene *Shp* was comparable compared with controls. Due to the indirect effect of FXR via SHP, it seems unlikely that FXR-SHP mediated the ineffective feedback response on bile salt synthesis. However, as *Cyp7A1* mRNA levels were unchanged in STZ-diabetes, the increase that was found in total bile salt synthesis (+50%) is most likely attributable to Cyp7A1 activity regulation at a post-transcriptional level. The 106% increased cholate synthesis in STZ diabetes was associated with a strongly increased Cyp8B1 expression. The increased *Cyp8B1* transcription may be attributed to increased PPARα activity due to increased fatty acid fluxes in the diabetic state. In STZ-diabetes, the elevated intestinal cholate absorption was not associated with changes in the expression of the apical sodium-dependent bile salt transporter (Asbt). Possibly this may implicate an overcapacity of this transport system, or the changed intestinal morphology in the diabetic state with an increased absorptive area and an increased mucosal width, may have caused

underestimation of the number of Asbt transporters.

In conclusion, in children and adolescents with type 1 diabetes and in STZ-induced diabetic rats alterations in cholesterol and bile salt metabolism associated with altered expression of transcription factors and transport proteins were found. Treatment with insulin did not completely reverse these effects. From recently described studies, it appears that not only the absence of insulin, but also the metabolic changes caused by insulin deficiency, such as elevated free fatty acids and acetoacetate concentrations, decreased intracellular glucose levels and increased advanced glycation end products, affect transcription factors and transport proteins involved in cholesterol and bile salt metabolism. Further studies are indicated to determine the factors that may induce the observed changes in expression of transcription factors and transport proteins. Furthermore, the implications of the observed changes in cholesterol and bile salt metabolism for children and adults with DM1 are yet to be defined. Obviously, an improvement of metabolic control will be the first target to lower plasma lipid levels and, thereby, the risk for atherosclerosis. Yet, as atherosclerosis in DM1 can already be found at an early age, recent guidelines for DM1 in children and adolescents advise additional pharmacological therapies. Statins are generally recommended as first choice treatment. On account of the above described studies, pharmacological therapies or dietary interventions inhibiting intestinal cholesterol absorption should be considered.

SAMENVATTING

Diabetes mellitus type 1 (DM1) is geassocieerd met microvasculaire (retinopathie, nefropathie, neuropathie) en macrovasculaire (hart- en vaatziekten) complicaties. Macrovasculaire aandoeningen zijn de belangrijkste doodsoorzaak bij patiënten met DM1. De mortaliteit, die bij DM1 wordt veroorzaakt door coronair lijden, stijgt snel na de leeftijd van 30 jaar. Atherosclerose bij DM1 is geassocieerd met de mate van metabole controle (hyperglycemie, HbA_{1c}), echter verscheidene studies en ook een recente meta-analyse tonen geen correlatie tussen atherosclerose en HbA_{1c}. Andere factoren die bij DM1 worden geassocieerd met het optreden van atherosclerose zijn roken, hypertensie, insuline resistentie, nefropathie, specifieke genetische factoren en stoornissen in de plasma lipiden concentraties. Deze veelheid aan variabelen wijst op een multifactoriële etiologie voor de toename van atherosclerose bij DM1. De studies in dit proefschrift beschrijven factoren die een rol kunnen spelen bij de ontwikkeling van atherosclerose bij DM1, waarbij gefocusseerd werd op het vet-, cholesterol- en galzout metabolisme. Verstoringen in postprandiaal vet- en cholesterolmetabolisme zijn onderzocht bij kinderen en adolescenten met DM1: aangenomen wordt dat het atherogene proces al op jonge leeftijd begint. Daarnaast zijn moleculaire mechanismen die ten grondslag liggen aan het verstoorde vet-, cholesterol- en galzout metabolisme onderzocht in een goed gekarakteriseerd diermodel voor DM1.

Hoofdstuk 1 geeft een algemeen overzicht over pathofysiologische processen die een rol spelen bij macrovasculaire complicaties en het lipoproteinen metabolisme met de effecten hierop van DM1. Beschikbare data uit studies naar atherosclerose en lipiden spiegels bij kinderen met DM1 zijn samengevat. Tevens worden het cholesterol en galzout metabolisme met de hierbij relevante receptoren, transport eiwitten en transcriptiefactoren kort beschreven.

Een vertraagde chylomicronen (CM) klaring heeft men bij volwassenen met DM1 geassocieerd met atherosclerose. Er zijn echter geen data beschikbaar bij kinderen en adolescenten met DM1. In **hoofdstuk 2** onderzochten we de CM klaring bij tieners met DM1 door na inname van een vetrijke maaltijd, die vitamine A en ^{13}C-oleaat bevatte, de $^{13}CO_2$ in de uitademinglucht en de plasma triglyceriden, retinyl palmitate en ^{13}C-oleaat concentraties te meten. Geen van deze testen gaven aanwijzingen voor een vertraagde CM klaring. Bij patiënten met DM1 waren de "nuchtere" triglyceriden en cholesterol concentraties positief gecorreleerd met de mate van metabole controle, weerspiegelt in HbA_{1c} percentages. De CM klaring bij tieners met DM1 toonde geen correlatie met HbA_{1c}, geslacht, cholesterol of triglyceriden gehalte. Het verschil in uitkomst tussen kinderen en volwassenen met DM1 kan mogelijk verklaard worden door een slechtere metabole controle bij de volwassen patiënten, of door een leeftijdsafhankelijke toename van de CM klaring, zoals bij gezonde volwassenen is beschreven.

Het LDL cholesterol kan bij DM1 verhoogd zijn door een verscheidenheid aan factoren. Bij ratten met door streptozotocine (STZ)-geïnduceerde diabetes (STZ-diabetes wordt gebruikt als diermodel voor DM1) bleek de cholesterol absorptie in de darm verhoogd te zijn vergeleken met controle ratten. Deze verhoogde cholesterol absorptie was geassocieerd met een verlaagde hepatische cholesterol synthese en onderdrukte hepatische LDL receptor expressie. Deze data suggereren dat hyperabsorptie van (dieet) cholesterol bij DM1 aanleiding geeft tot een vertraagde LDL klaring. Bij volwassenen met DM1 waren markers van cholesterol absorptie verhoogd en markers van cholesterol synthese verlaagd, wanneer de data werden vergeleken met gezonde individuen of met volwassenen met type 2 diabetes. Bij kinderen en adolescenten met DM1 is tot nu toe geen onderzoek naar de cholesterol absorptie en synthese verricht. In **hoofdstuk 3** werd de cholesterolsynthese en absorptie onderzocht bij kinderen met DM1. Bij kinderen met DM1 was een marker voor cholesterol synthese (plasma lathosterol/cholesterol ratio) significant lager dan bij kinderen zonder DM1, in overeenstemming met de recente studies bij volwassenen met DM1. De absolute waarden van de cholesterol absorptie markers (cholestanol, campesterol en sitosterol) waren significant hoger bij DM1 patiënten, echter, na correctie voor de plasma cholesterol concentratie werden deze ratio's vergelijkbaar. Het is mogelijk dat de niet nuchtere condities waaronder bloed werd afgenomen en het niet houden van een gecontroleerd dieet in onze studie, in tegenstelling tot de studies bij volwassenen met DM1, de uitkomst heeft beïnvloed. Bij de kinderen met DM1 en de controle kinderen was het vorderen van de leeftijd geassocieerd met stijgende cholesterol synthese markers en dalende cholesterol absorptie markers. De puberteit is geassocieerd met verlaagde insulinegevoeligheid, wat bij de kinderen met DM1 werd gereflecteerd door een met de leeftijd positief gecorreleerde dagelijkse insuline dosering (per kg lichaamsgewicht). Bij volwassenen met insulineresistentie is de cholesterol synthese verhoogd en de cholesterolabsorptie verlaagd t.o.v. controles. Een milde insuline resistentie tijdens de puberteit kan binnen onze studie hebben bijgedragen bij de stijging in cholesterol synthese en daling in absorptie met het vorderen van de leeftijd.

Om de onderliggende mechanismen van de beschreven veranderingen in het cholesterol metabolisme te bestuderen, onderzochten we de intestinale cholesterol absorptie en de cholesterol excretie in de gal bij ratten met door streptozotocine (STZ)-geïnduceerde diabetes (**hoofdstuk 4**). Bij STZ-diabetische ratten was de cholesterol excretie in de gal, in relatie tot de galzout en fosfolipiden excretie, verlaagd in vergelijking met controle ratten. De cholesterol absorptie was verhoogd bij STZ-ratten vergeleken met controles, zowel gemeten aan de hand van plasma plantsterolen concentraties als bij berekening van de cholesterol balans. De cholesterol absorptie en de cholesterol excretie in de gal normaliseerden gedeeltelijk na het toedienen van insuline.

Recente studies hebben aangetoond, dat de ATP bindende cassette (ABC) half-transporters, Abcg5 en Abcg8, een rol spelen in de uitscheiding van cholesterol in de gal en in het transport van cholesterol uit de enterocyt terug in het darm lumen. Bij STZ-diabetische ratten was de verlaagde cholesterol uitscheiding in de gal geassocieerd met een verlaagde expressie van de *Abcg5* en *Abcg8* genen in de lever. Tevens was de verhoogde cholesterol absorptie uit de darm geassocieerd met verlaagde intestinale expressie van *Abcg5* en *Abcg8* mRNA en een verlaagde Abcg5 eiwit expressie. Dit betekent dat de relatief verlaagde cholesterol excretie in de gal en de verhoogde cholesterol absorptie (deels) kan worden toegeschreven aan een verlaagde hepatische en instestinale *Abcg5* en *Abcg8* expressie. De transcriptie van zowel *Abcg5* als *Abcg8* wordt gereguleerd door de "Liver-X-receptor" (LXR). De expressie van het LXR gen wordt gestimuleerd door insuline. Bij de STZ-diabetische ratten zou de lagere insuline concentratie kunnen leiden tot een verlaagde LXR expressie en daardoor verlaagde Abcg5/g8 expressie. Echter, op mRNA niveau was de *Lxr* expressie niet significant verschillend van controles. Vrije vetzuren en acetoacetaat concentraties zijn verhoogd bij diabetes en kunnen de activatie van LXR door oxysterolen antagoneren. Wanneer hierdoor LXR minder actief is zal een verlaagde transcriptie van *Abcg5/g8* optreden. Concluderend is de verlaagde cholesteroluitscheiding in de gal en de verhoogde cholesterolabsorptie bij diabetes toe te schrijven aan een verlaagde Abcg5/g8 expressie in darm en lever, waarschijnlijk als gevolg van metabole verstoring van LXR activiteit.

Bij diabetes zijn meerdere veranderingen in het galzoutmetabolisme beschreven. Patiënten met type 2 diabetes toonden een vergrote galzoutpool en een verhoogde galzoutsynthese. Bij diermodellen voor **type 1** diabetes was de uitscheiding van galzouten en fosfolipiden in de gal duidelijk verhoogd en van glutathione verlaagd. De maximale secretiesnelheid (SRm) van galzouten, d.w.z., de maximale hoeveelheid van een bepaald galzout die per tijdseenheid in de gal kan worden uitgescheiden voordat er cholestase wordt geïnduceerd, bleek hoger te zijn bij diabetische ratten dan bij controles. De mechanismen die verantwoordelijk zijn voor deze veranderingen in galvorming zijn grotendeels onbekend. In **hoofdstuk 5** onderzochten we bij ratten met door STZ-geïnduceerde diabetes of verschillende ABC transport eiwitten in de canaliculaire membraan van de lever een rol spelen bij de veranderingen in galvorming. Een belangrijke bevinding was de verhoogde expressie van de fosfolipiden transporter Abcb4 (multidrug resistance P-glycoprotein type 2, of Mdr2-Pgp) op zowel mRNA (+105%) als eiwit (+530%) niveau. De verhoogde Abcb4 expressie was geassocieerd met een 520% toegenomen fosfolipiden uitscheiding in de gal. Behandeling met insuline leidde tot een verlaging van de fosfolipiden excretie en de Abcb4 expressie. Opvallend was, dat bij diabetes de expressie van de belangrijkste canaliculaire galzout transporter Abcb11 (bile salt export pump, Bsep) licht verhoogd was op mRNA niveau (+60%) maar onveranderd op eiwit niveau, terwijl de galzoutexcretie in de

gal met 260% was toegenomen. Bovendien bleek de SRm voor taurocholaat met 130% te zijn toegenomen, terwijl de SRm waarden bij met insuline-behandelde diabetische ratten tussen de controle en diabetes groep in lagen. De sterk toegenomen galzoutexcretie bij diabetische ratten met ongewijzigde Abcb11 eiwit expressie impliceert een behoorlijke overcapaciteit van dit transportsysteem. We hypothetiseren dat de sterk verhoogde fosfolipiden excretie, geassocieerd met de verhoogde Abcb4 expressie, de hepatische canaliculaire membraan beschermt tegen het etsende effect van de uitgescheiden galzouten. Dit faciliteert de verhoogde SRm voor taurocholaat bij STZ-diabetes. Eiwit concentraties van Abcc2 (multidrug resistance –associated protein 2, Mrp2), de transporter die betrokken is bij de uitscheiding van glutathione in de gal, bleek beduidend verlaagd bij diabetische ratten. Insuline behandeling gaf slechts een gering herstel van de Abcc2 expressie en glutathione uitscheiding in de gal. Glutathione is belangrijk voor het genereren van de galzout-onafhankelijke galflow. Bij zowel STZ-diabetische ratten als spontaan diabetische ratten is een verlaging van de galzout-onafhankelijke galflow beschreven en deze wordt waarschijnlijk veroorzaakt door de verlaagde Abcc2 expressie met daarbij een afgenomen glutathione uitscheiding. Wanneer dit mechanisme ook aanwezig is bij patiënten met DM1 zou dit de uitscheiding van geneesmiddelen in de gal kunnen verstoren.

In **hoofdstuk 6** werd de kinetiek van het galzout metabolisme onderzocht d.m.v. een isotoop verdunningsmethode. Stabiel gelabeld $^{2}H_{4}$-cholaat werd toegediend aan ratten met STZ-diabetes, controle ratten en STZ-diabetische ratten behandeld met insuline. De diabetische ratten hadden een met 535% verhoogde cholaat pool en een met 106% verhoogde cholaat synthese. Tevens bleek de berekende dagelijkse cholaat absorptie bij diabetes met 410% te zijn toegenomen. De toegenomen cholaat pool bij diabetes is daarmee het gevolg van zowel een verhoogde cholaat synthese en een meer efficiënte cholaat absorptie. Onder fysiologische omstandigheden leidt een verhoogde galzout absorptie tot een afname van de galzout synthese, echter bij diabetes lijkt dit niet op te treden. Insuline behandeling gaf slechts een gedeeltelijke normalisering van de kinetische data.

De reden waarom de verhoogde cholaat absorptie niet leidt tot verlaging van de galzoutsynthese is onbekend. Het galzout feedback mechanisme wordt (gedeeltelijk) gecontroleerd door de farnesoid X receptor (FXR). Bij STZ-diabetes vonden wij en anderen een verlaagde mRNA expressie van *Fxr,* wat is toegeschreven aan de overwegend lage intracellulaire glucose concentraties. Echter, de expressie van het target gen van FXR, small heterodimer partner (Shp), was vergelijkbaar met controles. Het lijkt dus onwaarschijnlijk dat FXR-SHP de ineffectieve feedback respons op de galzoutsynthese heeft gemedieerd. Omdat *Cyp7A1* mRNA spiegels niet gewijzigd waren bij STZ-diabetes, is de toename in totale galzoutsynthese (+50%) meest waarschijnlijk toe te schrijven aan regulatie van Cyp7A1 activiteit

op posttranscriptie niveau. De toename in cholaat synthese bij STZ-diabetes was geassocieerd met een sterk toegenomen *Cyp8B1* expressie. De verhoogde *Cyp8B1* transcriptie kan mogelijk worden verklaard door een verhoogde PPARα activiteit als gevolg van een verhoogd vetzuur aanbod in de diabetische situatie. De verhoogde cholaat absorptie bij diabetes was niet geassocieerd met veranderingen in expressie van de apicale natrium-afhankelijke galzout transporter (Asbt). Mogelijk heeft dit transportsysteem een grote overcapaciteit of heeft de gewijzigde darm morfologie bij diabetes, waarbij het absorptie oppervlak en de mucosa breedte zijn toegenomen, geleid tot een onderschatting van het aantal Asbt transporters.

Concluderend werden bij kinderen en adolescenten met type 1 diabetes en bij diabetische ratten veranderingen in het cholesterol en galzout metabolisme gevonden, die waren geassocieerd met gewijzigde expressie van belangrijke transcriptiefactoren en transport eiwitten. Behandeling met insuline gaf geen volledige omkering van deze effecten. Uit recent beschreven studies blijkt dat niet alleen de afwezigheid van insuline, maar ook de metabole veranderingen die door insulinetekort worden veroorzaakt, zoals verhoogde vrije vetzuur en acetoacetaat concentraties, verlaagde intracellulaire glucose concentraties en verhoogde AGEs (advanced glycation end products), de transcriptiefactoren en transport eiwitten, die betrokken zijn bij het cholesterol- en galzout metabolisme beïnvloeden. Verder onderzoek op dit gebied is dus geïndiceerd. Bovendien dienen de implicaties van de gevonden wijzigingen in het cholesterol en galzout metabolisme voor kinderen en volwassenen met DM1 te worden opgehelderd. Het is voor de hand liggend dat een verbetering van de metabole controle altijd het eerste doel is, om op deze wijze de plasma lipiden concentraties te verlagen en daardoor het risico op atherosclerose te verminderen. Echter, daar atherosclerose bij DM1 al op jonge leeftijd kan worden aangetoond, worden in de recente richtlijnen voor DM1 bij kinderen en adolescenten additionele farmacologische therapieën geadviseerd voor de behandeling van dyslipidemie. Hierbij wordt als eerste de voorkeur gegeven aan statines. Naar aanleiding van bovenstaande beschreven studies zouden farmacologische therapieën of dieet interventies gericht op het beperken van de cholesterol absorptie in de darm overwogen moeten worden.

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Willie

CURRICULUM VITAE

Willemijntje Maria (Willie) Bakker-van Waarde werd op 3 juni 1965 geboren te Ouddorp (Goeree Overflakkee, Z-H). Na het behalen van haar Atheneum-B diploma aan de Rijksscholengemeenschap Goeree Overflakkee te Middelharnis in 1982, startte zij met de studie Geneeskunde aan de Rijksuniversiteit te Leiden. In 1986 behaalde zij haar doctoraal examen en in 1989 haar artsexamen (cum laude). Van 1 juli 1989 tot 1 oktober 1989 was zij werkzaam als arts-assistent interne geneeskunde in het van Weel Bethesda ziekenhuis te Dirksland. Vanaf 1 oktober 1989 startte zij haar werkzaamheden binnen de kindergeneeskunde als arts-assistent niet in opleiding in het Academisch Ziekenhuis Groningen. Op 1 juni 1991 startte zij met de opleiding tot kinderarts, de eerste 2 jaar in het Medisch Spectrum Twente te Enschede (waarnemend opleider destijds drs. A.N. Bosschaart) en vervolgens in de Beatrix Kinderkliniek van het Academisch Ziekenhuis Groningen (opleider destijds Prof. Dr. H.S.A. Heymans). Vanaf 1 februari 1996 werkte zij als kinderarts-supervisor binnen de Beatrix Kinderkliniek van het AZG. Op 1-10-1996 startte zij met haar fellowship kinderendocrinologie (Hoofd: Drs C.W. Rouwé), aanvankelijk afwisselend met de functie kinderarts-supervisor. Vanaf 1998 was zij fulltime fellow kinderendocrinologie. Er werd in deze periode gestart met haar promotie onderzoek binnen het Centrum voor Lever-, Stofwisseling en Metabole ziekten (Hoofd: Prof. Dr. F. Kuipers), waarvan de resultaten zijn beschreven in dit proefschrift. Registratie tot kinderarts-endocrinoloog vond plaats op 14-03-2001. Sinds die tijd is ze werkzaam als kinderarts-endocrinoloog binnen de Beatrix Kinderkliniek van het Universitair Medisch Centrum Groningen. Willie is getrouwd met Martin Bakker. Samen hebben ze 2 dochters: Elianne (2002) en Lotte (2003).

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